Charakterisierung des Transkriptionsfaktors CcpN aus *Bacillus subtilis*

Dissertation

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Abkürzungsverzeichnis

Acetyl-CoA	Acetyl-Coenzym A
ADP	Adenosindiphosphat
ATP	Adenosintriphosphat
B. subtilis	*Bacillus subtilis*
bp	Basenpaar
cAMP	zyklisches Adenosinmonophosphat
CBS	Cystathion-β-Synthetase
CcpA	catabolite control protein A
CcpN	catabolite control protein of gluconeogenic genes
CCR	carbon catabolite repression
CD	Circular-Dichroismus
cre	catabolite responsive element
DNA	Desoxyribonucleinsäure
DNase I	Desoxyribonuclease I
E. coli	*Escherichia coli*
GapB	Glycerinaldehyd-3-Phosphat-Dehydrogenase B
His	Histidin
HPr	histidine-containing protein
K_D	Dissoziationskonstante
mRNA	Messenger-RNA
nt	Nucleotid
PckA	Phosphoenolpyruvat-Carboxykinase
PTS	Phosphotransferase-System
RNA	Ribonucleinsäure
RNAP	RNA-Polymerase
Ser	Serin
SR1	Small RNA 1
ThyB	Thymidylatsynthase B
z. B.	zum Beispiel
α-CTD	C-terminale Domäne der α-Untereinheit
α-NTD	N-terminale Domäne der α-Untereinheit

1. Einleitung

1.1. Transkriptionsregulation in Prokaryoten

1.1.1. Die Transkriptionsinitiation

Bakterien sind im Laufe ihres Daseins mit einer Vielzahl unterschiedlicher Umweltbedingungen konfrontiert, auf welche sie angemessen reagieren müssen. Während geringe Schwankungen z. B. der Osmolarität oder der Konzentration intrazellulärer Metabolite durch die Aktivität entsprechender Porine oder Stoffwechselenzyme ausgeglichen werden können, bedürfen größere und langfristig anhaltende Schwankungen einer intensiveren Regulation. Da auf sich ändernde Umweltbedingungen in der Regel mit einer Änderung der Proteinzusammensetzung der Zelle reagiert werden muss, steht Bakterien hier nur eine begrenzte Anzahl an Mechanismen zur Verfügung. Es kann zum einen die Menge an Protein reguliert werden, entweder durch Kontrolle der Syntheserate oder durch Kontrolle des Abbaus, zum anderen kann die Menge an mRNA für das entsprechende Protein reguliert werden. Letzteres ist wiederum entweder durch Regulation der Synthese oder des Abbaus möglich, wobei ersteres die deutlich ökonomischere Variante darstellt, da bereits die Synthese einer mRNA einen Energieaufwand für die Zelle bedeutet.

Die Regulation der Synthese einer RNA findet in der Regel während der Transkriptionsinitiation, seltener während der Elongation statt. Die Initiation der Transkription ist ein komplexer Prozess, der aus einer sequentiellen Abfolge mehrerer Schritte besteht (Record *et al.*, 1996; Abbildung 1). Im ersten Schritt bindet die bakterielle RNA-Polymerase (RNAP), ein aus mehreren Untereinheiten bestehendes Enzym, an eine Promotor-Region. Die Polymerase selbst besteht aus einer β- und einer β'-Untereinheit, die das katalytische Zentrum bilden (Korzheva *et al.*, 2000). Die Assemblierung dieser beiden Untereinheiten wird durch die N-terminale Domäne der α-Untereinheiten (α-NTD) unterstützt (Blatter *et al.*, 1994), die C-terminale Domäne (α-CTD) besitzt unterstützende Funktion bei der Promotorerkennung und -bindung (Gourse *et al.*, 2000). Der σ-Faktor schließlich ist für die Erkennung der Promotor-Sequenz und die Rekrutierung der RNAP an den Promotor verantwortlich (Wösten, 1998). Neben diesen notwendigen Untereinheiten gibt es mehrere, die mit der RNAP assoziiert sein können, ihre Funktion unterstützen, aber für eine Promotorerkennung und Transkription nicht zwingend notwendig sind. Beispiele hierfür sind die ω-Untereinheit, die als Chaperon die korrekte Faltung der β'-Untereinheit unterstützt

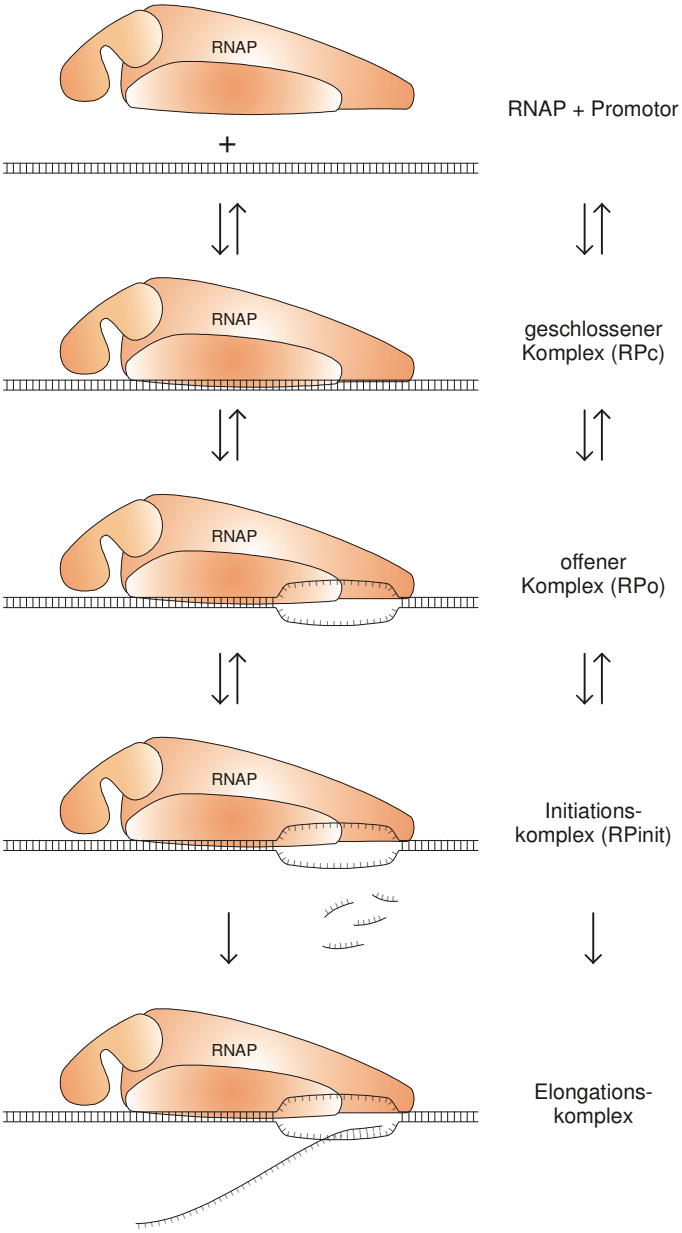


Abbildung 1:

Phasen der Transkriptionsinitiation

Die verschiedenen Komplexe, die im Zuge des Transkriptionsnitiations-prozesses gebildet werden, sind schematisch dargestellt. Bis auf den Übergang in den Elongationskomplex sind alle Schritte reversibel.

(Hampsey, 2001), oder die δ-Untereinheit von *Bacillus subtilis*, die die Transkriptionseffizienz der Polymerase erhöht und eine bislang nicht näher aufgeklärte Rolle während der Sporulation spielt (Gao & Aronson, 2004). Die Promotor-Region besteht aus vier Sequenzelementen: Einer –10-Region und einer –35-Region, die an jedem Promotor vorhanden sind und von den Domänen 2 und 4 des σ-Faktors erkannt werden (Busby & Ebright, 1994; Murakami *et al.*, 2002). Für diese Regionen wurden die Konsensus-Sequenzen TATAAT (–10-Region) und TTGACA (–35-Region) ermittelt. Des Weiteren können eine erweiterte –10-Region, ein drei bis vier Basenpaare langes Motiv der Sequenz TG_n (Sanderson *et al.*, 2003), sowie sogenannte UP-Elemente, AT-reiche Bereiche upstream der –35-Region (Ross *et al.*, 2001), vorhanden sein. Durch Bindung der RNAP an den Promotor entsteht der sogenannte geschlossene Komplex (RPc). Dieser Komplex geht danach in einen

Komplex über, bei dem die RNAP fester an die DNA gebunden ist (RPc2). Anschließend findet ein Aufschmelzen der DNA im Bereich von ungefähr –10 bis +4 statt, das durch die Domäne 2 des σ-Faktors katalysiert wird, es entsteht der offene Komplex (RPo) (Tsujikawa *et al.*, 2002). Dieser Komplex geht dann durch Einbau der ersten Nukleosidtriphosphate in den Initiationskomplex (RPinit) über, welcher temporär kurze, 3-10 nt lange abortive Transkripte erzeugen kann, die aus fehlgeschlagenen Versuchen der RNAP, den Promotor zu verlassen, resultieren. Gelingt das Verlassen des Promotors schließlich, geht die RNAP in den Elongationskomplex über. Dieser letzte Schritt ist irreversibel, während alle vorhergehenden Schritte vollständig reversibel sind. An jedem Promotor ist jeder Übergang eines Komplexes in den anderen durch eine charakteristische Aktivierungsenergie gekennzeichnet, wodurch der Schritt mit der höchsten Aktivierungsenergie zum geschwindigkeitsbestimmenden Schritt wird.

Die Regulation des Transkriptionsinitiationsprozesses kann nun nicht nur an jedem dieser Schritte ansetzen, sondern auch in jede beliebige Richtung erfolgen: Proteine, die die Aktivierungsenergie eines bestimmten Schrittes senken, wirken als Aktivatoren, solche, die die Aktivierungsenergie erhöhen, wirken als Repressoren.

1.1.2. Regulation durch Promotor-Selektivität

Transkriptionsregulation kann auf einer basalen Ebene durch die Sequenz eines Promotors stattfinden. Promotoren, deren Elemente nahe an der Konsensus-Sequenz sind, sind in der Regel effizienter als solche mit stark abweichenden Sequenzen. Da diese Regulation allerdings statisch in der DNA-Sequenz verankert ist, dient sie lediglich dazu, die RNAP ungleichmäßig auf verschiedene Promotoren zu verteilen und so eine generelle Kontrolle der Transkriptmenge zu erreichen.

Einen Spezialfall unter den Regulatoren der Transkription stellen die σ-Faktoren dar. Verschiedene Organismen besitzen eine unterschiedliche Menge an σ-Faktoren, von einem einzigen bei *Mycoplasma genitalium* bis hin zu 63 bei *Streptomyces coelicolor* (Gruber & Gross, 2003). Jeder σ-Faktor besitzt unterschiedliche Voraussetzungen bezüglich der Sequenz der –10- und –35-Region (die oben angegebenen Konsensussequenzen gelten für den Haupt-σ-Faktor), wodurch die RNAP durch verschiedene σ-Faktoren gezielt zu einem bestimmten Satz an Promotoren geführt werden kann. Eine Regulation der σ-Faktor-Aktivität kann zum einen durch dessen Synthese und Abbau stattfinden, zum anderen durch sogenannte Anti-σ-

Faktoren. Diese Proteine binden einen spezifischen σ-Faktor und verhindern so, dass dieser die RNA-Polymerase binden kann (Hughes & Mathee, 1998).

1.1.3. Regulation durch Transkriptionfaktoren

1.1.3.1. Aktivierung

Aktivierung der Transkriptionsinitiation geschieht in der Regel während der Bildung des geschlossenen Komplexes (Abbildung 2). Hierbei sind Promotoren, deren Sequenz stark vom Konsensus abweicht, besonders prädestiniert, da diese eine schwache Affinität für die RNAP und somit nur eine geringe Promotorstärke besitzen. Generell lassen sich Aktivatoren in drei Klassen einteilen: Aktivatoren der Klasse I rekrutieren die RNAP an den Promotor, indem sie mit der α-CTD interagieren. Solche Aktivatoren binden upstream der –35-Region, besitzen aber keine engen Voraussetzungen hinsichtlich der Operator-Position, da die α-CTD durch einen flexiblen Linker mit der RNAP verbunden ist. Beispiele für solche Aktivatoren sind das

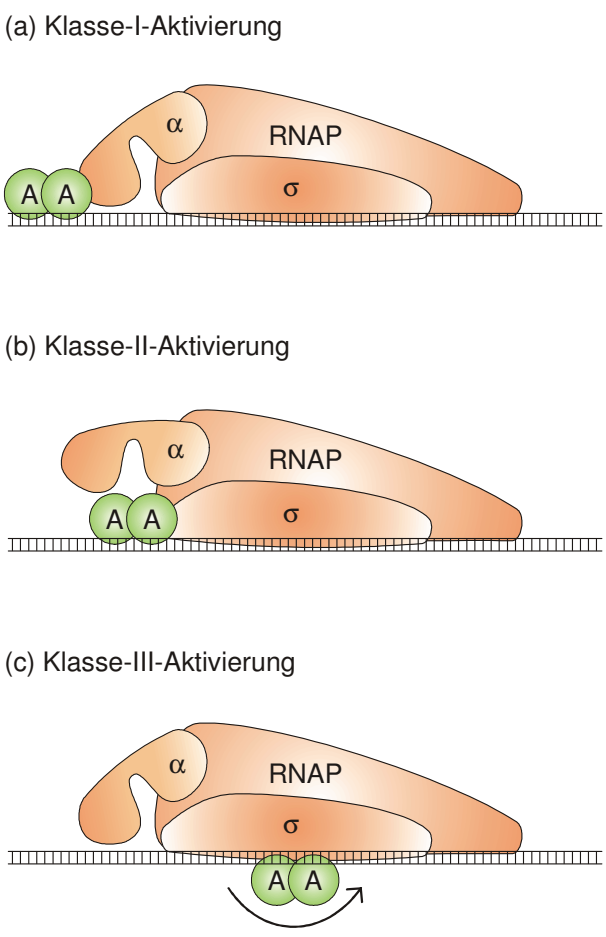


Abbildung 2:

Mechanismen der Transkriptionsaktivierung

Aktivatoren sind mit einem A gekennzeichnet und als Dimer dargestellt, da sie oft als Dimer arbeiten, RNAP stellt die RNA-Polymerase dar, α die α-Untereinheit, σ den σ-Faktor.

(a): Klasse-I-Aktivierung. Der Aktivator bindet upstream des Promotors und rekrutiert die RNA-Polymerase durch Kontakte zu den α-CTDs.

(b): Klasse-II-Aktivierung. Der Aktivator bindet an Position –41,5 und interagiert mit Domäne 4 des σ-Faktors.

(c): Aktivierung durch Konformationsänderung. Der Aktivator bindet zwischen der –35- und –10-Region und richtet diese neu zueinander aus, sodass die RNA-Polymerase binden kann.

cAMP-Rezeptor-Protein CRP am *lac*-Promotor von *E. coli* (Ebright, 1993) oder das Protein p4 des *B. subtilis*-Phagen Φ29 am A3-Promotor (Nuez *et al.*, 1992; Mencía *et al.*, 1996). Bei Aktivatoren der Klasse II handelt es sich im Proteine, die mit der Domäne 4 des σ-Faktors interagieren (Dove *et al.*, 2003). Da der σ-Faktor in seiner Position nicht flexibel ist, befinden sich Operatoren für solche Aktivatoren in der Regel um die Position –41,5. Ein Beispiel für einen solchen Aktivator wäre das CI-Protein des Bakteriophagen λ am λ-PRM-Promotor (Nickels *et al.*, 2002). Es sind auch Fälle bekannt, bei denen Klasse-II-Aktivatoren anstatt des σ-Faktors die α-NTD kontaktieren (Busby & Ebright, 1997). Aktivierung der Klasse III findet man an Promotoren, bei denen die –10- und die –35-Region für die Bindung der RNAP nicht optimal zueinander ausgerichtet sind, z. B. durch eine zu große Spacer-Region. Durch Bindung des Aktivators wird die räumliche Struktur der DNA verändert und die bindungsrelevanten Promotorelemente in eine günstigere räumliche Position gebracht. Mitglieder aus der MerR-Familie von Aktivatoren, wie z. B. BmrR, nutzen diesen Mechanismus der Aktivierung (Heldwein *et al.*, 2001; Brown *et al.*, 2003). Alternativ werden der Klasse III auch solche Aktivatoren zugerechnet, die weit upstream der RNAP binden und mit dieser über die Ausbildung eines DNA-Loops interagieren. Die Bildung des DNA-Loops kann dabei durch einen weiteren Aktivator begünstigt werden.

Einen Sonderfall bilden Promotoren, die von σ^{54} von *E. coli* erkannt werden. Diese sind in der Regel durch die geringe Stabilität des offenen Komplexes limitiert und benötigen Aktivatoren, die spezifisch den offenen Komplex stabilisieren (Buck *et al.*, 2000).

1.1.3.2. Repression

Im Gegensatz zu Aktivatoren, die fast ausschließlich die Bildung des geschlossenen Komplexes fördern, nutzen Repressoren jeden Schritt des Transkriptionsinitiationsprozesses (Abbildung 3). Die einfachste Möglichkeit der Repression besteht in einer sterischen Behinderung der RNAP-Bindung, also der Bildung des geschlossenen Komplexes, welche meist durch eine Überlappung der Operator-Sequenzen mit der –10- oder –35-Region erreicht wird. Als Bespiele seien hier der am O1-Operator des *lac*-Promotors gebundene LacI-Repressor (Schlax *et al.*, 1995) oder auch das bereits als Aktivator vorgestellte Φ29-Protein p4 genannt, das am A2b-Promotor die RNAP-Bindung reprimieren kann (Rojo & Salas, 1991). Neben direkter sterischer Interferenz kann die RNAP-Bindung auch auf andere Arten verhindert werden. Der GalR-Repressor kann an mehreren Stellen binden, die nicht mit der Promotor-Region überlappen, und anschließend durch Interaktion der an verschiedenen

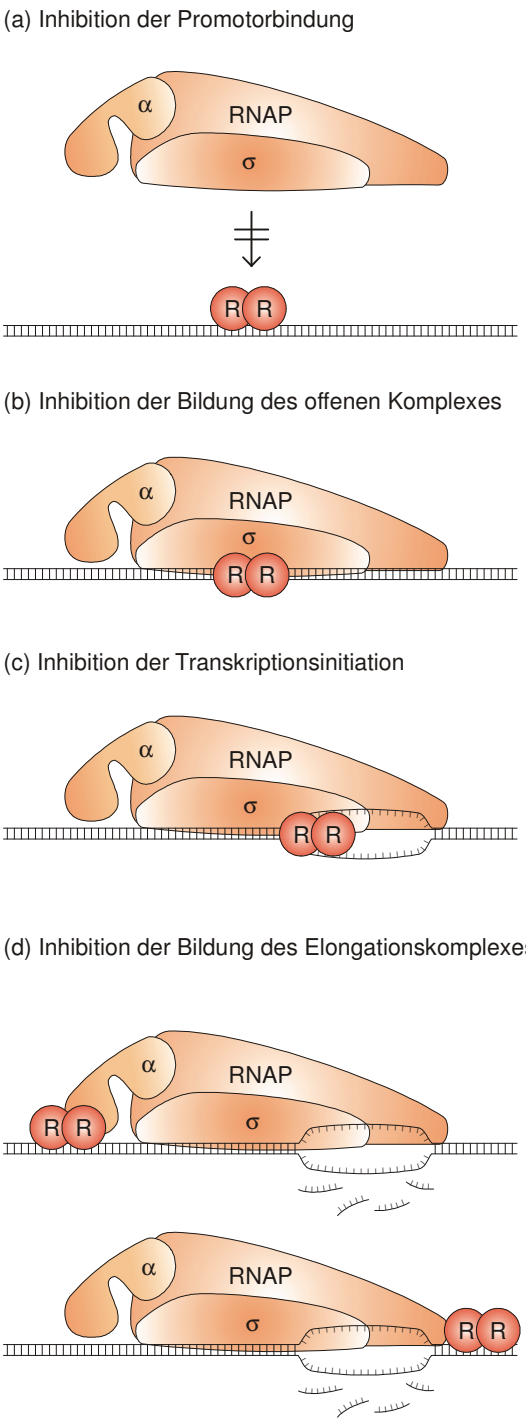


Abbildung 3:

Mechanismen der Transkriptionsrepression

Repressoren sind mit einem R gekennzeichnet und als Dimer dargestellt, da sie oft als Dimer arbeiten, RNAP stellt die RNA-Polymerase dar, α die α-Untereinheit, σ den σ-Faktor

(a): Inhibierung der RNAP-Bindung. Durch Bindung an die Promotorregion blockiert der Repressor die Bindungsstelle für die RNA-Polymerase.

(b): Inhibierung der Bildung des offenen Komplexes. Trotz Überlappung der Bindungsstellen können RNAP und Repressor gleichzeitig binden, jedoch erlaubt die Anwesenheit des Repressors keine Bildung eines stabilen offenen Komplexes.

(c): Inhibierung der Transkriptionsinitiation. Der Repressor und die RNAP können gleichzeitig binden und es wird auch ein stabiler offener Komplex gebildet. Die Anwesenheit des Repressors inhibiert allerdings jegliche Transkription, es werden weder abortive noch komplette Transkripte gebildet.

(d): Inhibierung der Bildung eines Elongations-komplexes. Der Repressor arretiert die RNAP am Promotor, entweder durch direkte Interaktion mit einer RNAP-Untereinheit (oben) oder durch Bildung einer Blockade downstream der RNAP (unten). Abortive Transkripte werden gebildet, die RNAP kann den Promotor jedoch nicht verlassen.

Operatoren gebunden Repressoren ein DNA-Loop bilden, welches die Bindung der RNAP verhindert (Choy & Adhya, 1996). Eine weitere Möglichkeit ist die Funktion als Anti-Aktivator, welche z. B. beim CytR-regulierten *deo*-Promotor in *E. coli* verwirklicht wurde. Hier bindet der CytR-Repressor mit Hilfe von zwei als Aktivator gebundenen CRP-Dimeren und verhindert durch diese Bindung deren Interaktion mit der α-CTD, wodurch die RNAP nicht an den Promotor rekrutiert werden kann (Valentin-Hansen *et al.*, 1996; Shin *et al.*, 2001).

An zahlreichen Promotoren wird der Übergang vom geschlossenen zum offenen Komplex durch Repressoren beeinflusst. Diese Repressoren können Operatoren besetzen, die direkt mit dem Promotor überlappen, jedoch trotzdem die gleichzeitige Bindung von Repressor und RNAP erlauben, wie zum Beispiel der MerR-Repressor am *merT*-Promotor, für den *in vitro* und *in vivo* eine Inhibierung der Bildung des offenen Komplexes gezeigt wurde (Heltzel *et al.*, 1990), oder das Spo0A-Protein aus *B. subtilis* am *abrB*-Promotor (Greene & Spiegelman, 1996). Es existieren allerdings auch Repressoren der Bildung des offenen Komplexes, deren Operatoren nicht mit der RNAP-Bindungsstelle überlappen, wie der Repressor KorB am *korABF*-Promotor (Williams *et al.*, 1993). Einen Spezialfall stellen hier wieder σ^{54}-kontrollierte Promotoren dar, bei denen, wie oben beschrieben, die Bildung des offenen Komplexes durch Aktivatoren gewährleistet werden muss. An diesen Promotoren existiert ein Repressionsmechanismus, bei dem ein Repressor zwischen Aktivator und RNAP bindet, dadurch eine DNA-Biegung induziert, was letztendlich die Interaktion zwischen Aktivator und RNAP verhindert. Als Beispiele hierfür seien der Nac-Regulator aus *Klebsiella aerogenes* (Feng *et al.*, 1995) oder auch der globale Regulator CcpA aus *B. subtilis* bei der Regulation des *lev*-Operons genannt (Martin-Vestraete *et al.*, 1995).

Für eine Repression des folgenden Schrittes, der Bildung des Initiationskomplexes und der damit einhergehenden abortiven Transkripte, gibt es nur wenige Belege in der Literatur. Ein derartiger Mechanismus wurde für das H-NS-Protein am *rrnB* P1-Promotor (Schröder & Wagner, 2000) und das FIS-Protein am *gyrB*-Promotor berichtet (Schneider *et al.*, 1999). In beiden Fällen wurde gezeigt, dass offene Komplexe gebildet werden, jedoch waren keinerlei Transkripte nachweisbar.

Deutlich häufiger findet man eine Inhibition des Überganges zum Elongationskomplex. Hierbei werden zwar abortive Transkripte gebildet, allerdings gelingt es der RNAP nicht, die Bindung an den Promotor zu überwinden. Dieses Phänomen tritt auch auf, wenn die Promotorsequenz in allen 4 relevanten Bereichen nahe dem Konsensus ist oder diesem entspricht. Dies führt zwar zu einer effizienten Rekrutierung der Polymerase an den Promotor, allerdings gelingt es ihr nicht, den Promotor wieder zu verlassen (Ellinger *et al.*, 1994). Natürlich vorkommende Promotoren stellen deswegen meist einen Kompromiss zwischen effizienter RNAP-Bindung und effizientem Promotor-Escape dar. Repressoren, die meist hochspezifisch und auch fest an die DNA binden, können den oben angeführten Effekt nachahmen, indem sie mit der RNAP interagieren und diese so am Promotor fixieren. Für diesen Mechanismus gibt es nur wenige Beispiele, wie das bereits als Aktivator und Repressor vorgestellte Protein p4 des Phagen Φ29 (Monsalve *et al.*, 1996). Es kann,

zusätzlich zu den bereits genannten Effekten, die RNAP durch Interaktion mit der α-CTD am A2c Promotor stabilisieren und so einen Übergang zum Elongationskomplex verhindern. Auch der Gal-Repressor funktioniert nach diesem Prinzip (Choy *et al.*, 1995). Eine weitere Möglichkeit der Verhinderung der Bildung eines Elongationskomplexes ist die Bindung eines Repressors downstream der +1-Position. Dieser Repressor muss nicht mit der RNAP interagieren, sondern stellt durch seine feste Bindung eine Blockade dar, die die RNAP nicht überwinden kann. Man findet diese Art der Repression beim *treP*-Gen in *B. subtilis*, welches von CcpA negativ reguliert wird (Ujiie *et al.*, 2009). Des weiteren wurde durch ein artifizielles Konstrukt, bei dem ein *lac*-Operator downstream eines nicht von LacI regulierten Promotors gebracht wurde, gezeigt, dass diese Art der Repression theoretisch von jedem hinreichend fest bindenden Repressor vermittelt werden kann (Lopez *et al.*, 1998).

1.1.4. Regulation der Transkriptionsregulatoren

Da Transkriptionsfaktoren die Transkription nur unter bestimmten Bedingungen beeinflussen sollen, muss auch ihre Aktivität reguliert werden. Dies kann wiederum durch Regulation von Synthese und Abbau geschehen. Allerdings sind ökonomischere Möglichkeiten vorhanden, die außerdem in einem kürzeren Zeitraum zum Tragen kommen. Viele Transkriptionsfaktoren sind Teil eines bakteriellen Zweikomponentensystems, das aus einer Sensor-Kinase und einem Response-Regulator, dem Transkriptionsfaktor, besteht. Die Kinase detektiert dabei Signale aus der Umwelt oder auch dem Zytoplasma und phosphoryliert daraufhin den Regulator, welcher erst durch die Phosphorylierung an die DNA binden kann oder aber bereits an die DNA gebunden ist und erst durch die Phosphorylierung aktiv wird, wie z. B. DeoR aus *B. subtilis* (Zeng & Saxild, 1999). Eine weitere Möglichkeit ist die Bindung kleiner Effektor-Moleküle, die von Ionen, wie z. B. Ni^{2+} im Falle von NikR aus *E. coli* (Fauquant *et al.*, 2006) bis hin zu verschiedenen Metaboliten reichen kann. Auch hier kann wieder durch Bindung oder Abwesenheit des Liganden die Bindung des Repressors an die DNA induziert oder aber ein bereits gebundener Regulator in eine aktive Konformation übergehen.

1.2. Katabolitrepression in *B. subtilis*

Der Metabolismus der meisten Bakterien ist dazu ausgelegt, eine Vielzahl von möglichen Nahrungsquellen zu verwerten. Allerdings sind für die Erschließung vieler dieser

Nahrungsquellen hochspezialisierte Enzyme notwendig, die nicht im Basismetabolismus verwendet werden. Um trotz der Option, eine möglichst große Vielfalt an Substraten zu verwerten, ökonomisch sinnvoll zu arbeiten, gibt es Kontrollmechanismen in Prokaryoten, die sicherstellen, dass bevorzugte Nahrungsquellen, also solche, die mit möglichst geringem Zusatzaufwand genutzt werden können, primär verwertet werden. Bei den meisten Organismen ist diese bevorzugte Nahrungsquelle aufgrund der Möglichkeit, sie einfach in die Glycolyse einfließen zu lassen, Glucose. Der Mechanismus, der die bevorzugte Verwertung von Glucose bei der gleichzeitigen Abschaltung alternativer Katabolismuswege vermittelt, wird Katabolitrepression (CCR, carbon catabolite repression) genannt. In *E. coli*, dem wohl wichtigsten Gram-negativen Modellorganismus, wurde cAMP, das sich bei Glucosemangel in der Zelle anreichert, als Signal für diesen Mechanismus identifiziert (Perlman *et al.*, 1969). Dieses kann vom cAMP-Rezeptor-Protein (CRP) gebunden werden, das als Aktivator alternative Stoffwechselwege induziert. Um eine gewisse Selektivität bezüglich der Aktivierung dieser Stoffwechselwege zu erreichen, besitzen diese in der Regel noch einen Repressor, der erst in Anwesenheit der entsprechenden Nahrungsquelle die Expression der entsprechenden Gene erlaubt, wie z. B. der LacI-Repressor am *lac*-Operon, der bei Anwesenheit von Lactose im Medium von der DNA gelöst wird (Lewis, 2005).

1.2.1. Elemente des CCR-Systems in *B. subtilis*

In *B. subtilis* hingegen, dem bedeutendsten Gram-positiven Modellorganismus, konnte auch unter Glucose-Mangel-Bedingungen weder eine signifikante Konzentration von cAMP in der Zelle noch ein CRP-ähnliches Protein nachgewiesen werden, was das *E.-coli*-Modell der Katabolitrepression ausschließt. CCR wird in *B. subtilis* stattdessen von einem *cis*-aktiven Element, der *cre*-Sequenz (catabolite responsive element) (Nicholson *et al.*, 1987; Weickert *et al.*, 1990), und einem *trans*-aktiven Faktor, CcpA (catabolite control protein A) (Henkin *et al.*, 1991), vermittelt. CcpA wirkt in der Regel als Repressor, und seine spezifische Bindung an die *cre*-Sequenz konnte auch *in vitro* bestätigt werden (Fujita *et al.*, 1995). Eine Gesamtübersicht über das Katabolitrepressionssystem in *B. subtilis* ist in Abbildung 4 dargestellt. Insgesamt wurden bisher über 50 *cre*-Sequenzen im Genom von *B. subtilis* identifiziert und deren Beteiligung an der Regulation von Genen und Operons experimentell bestätigt. Bioinformatische Analysen zeigten das Vorhandensein von 150 potentiellen *cre*-Sequenzen, die zusammen ca. 300 Gene regulieren. Aus den 50 experimentell verifizierten *cre*-Sequenzen wurde die Konsensus-Sequenz WTGAAARCGYTTWNN abgeleitet (Miwa &

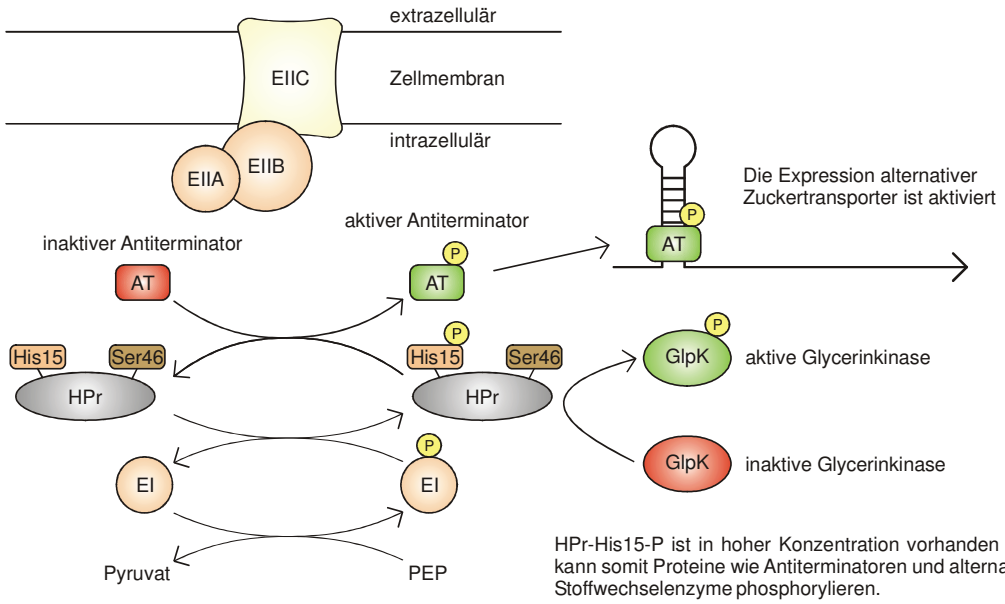
Fujita, 1990), wobei festgestellt wurde, dass die meisten der natürlich vorkommenden *cre*-Sequenzen geringe Abweichungen vom Konsensus aufweisen und diese Abweichungen für ihre jeweilige Funktion notwendig sind (Miwa *et al.*, 2000). Da der an die *cre*-Sequenz bindende Transkriptionsfaktor CcpA konstitutiv exprimiert wird (Miwa *et al.*, 1994), wurde schnell klar, dass es noch mindestens einen weiteren Faktor geben muss, der in die Katabolitrepression bei *B. subtilis* involviert ist.

Dieser Faktor wurde einige Zeit später als das Protein HPr (histidine-containing protein) identifiziert (Deutscher *et al.*, 1994). HPr erfüllt mehrere komplexe Aufgaben in der Stoffwechselregulation von *B. subtilis*. Das Protein selbst besitzt 2 Phosphorylierungsstellen, eine am Histidin 15 und eine am Serin 46. Beide Phosphorylierungszustände schließen sich gegenseitig aus, d. h. es kann immer nur eine der beiden Positionen phosphoryliert sein. Die Position His15 spielt eine wichtige Rolle beim Transport von Zuckern in die Zelle, da von dort ein Phosphat-Rest auf einen Zuckertransporter des Phosphoenolpyruvat-abhängigen Zucker-Phosphotransferase-Systems (PTS) und anschließend auf den in die Zelle transportierten Zucker übertragen werden kann. Die über dieses System transportierten Zucker werden kurz PTS-Zucker genannt und stellen in der Regel die Hauptnahrungsquelle von *B. subtilis* dar (Postma *et al.*, 1993; Reizer, 1996). Wenn diese Zucker nicht zur Verfügung stehen, kann am His15 phosphoryliertes HPr auch Enzyme wie die Glycerinkinase und diverse Antiterminatoren alternativer Stoffwechselgene phosphorylieren und damit aktivieren (Tortosa & Le Coq, 1995; Schnetz *et al.*, 1996; Darbon *et al.*, 2002).

1.2.2. CcpA-abhängige Katabolitrepression

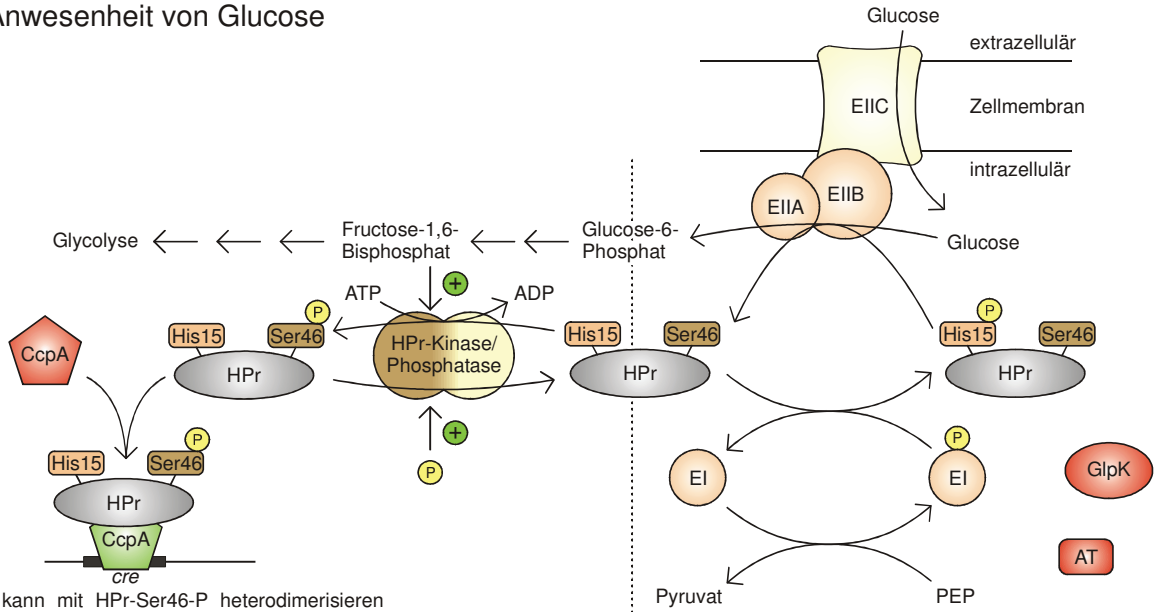
Eine Phosphorylierung von HPr am Ser46 hat dagegen eine völlig andere Funktion. Die Phosphorylierung an dieser Position wird durch die HPr-Kinase/Phosphatase katalysiert, die wiederum von steigenden Konzentrationen Fructose-1,6-Bisphosphat aktiviert wird, also immer dann, wenn ausreichend Nährstoffe und damit Glycolyseintermediate vorhanden sind (Reizer *et al.*, 1998). In diesem Phosphorylierungszustand (HPr-Ser46-P) ist HPr in der Lage, mit dem Transkriptionsfaktor CcpA zu interagieren und dessen Fähigkeit, *cre*-Elemente zu binden, signifikant zu erhöhen (Deutscher *et al.*, 1995; Jones *et al.*, 1997). Die Phosphorylierung am Ser46 ist reversibel und kann durch die Phosphatase-Aktivität der HPr-Kinase/Phosphatase entfernt werden. Die Phosphatase-Aktivität wird durch Phosphat-Ionen stimuliert, die auf einen niedrigen intrazellulären ATP-Spiegel und dadurch schlechte metabolische Bedingungen hindeuten (Hanson *et al.*, 2002). Dies erklärt auch, wie das

(a) Abwesenheit von Glucose



HPr-His15-P ist in hoher Konzentration vorhanden und kann somit Proteine wie Antiterminatoren und alternative Stoffwechselenzyme phosphorylieren.

(b) Anwesenheit von Glucose



CcpA kann mit HPr-Ser46-P heterodimerisieren und die Transkription an Promotoren katabolischer Operons, die *cre*-Elemente enthalten, regulieren.

2: In der späten Phase der Glucoseverwertung wird HPr an Ser46 phosphoryliert und kann dadurch mit CcpA Heterodimere bilden. HPr-Ser46-P kann nicht mehr am His15 phosohoryliert werden.

1: In der frühen Phase der Glucoseverwertung wird HPr an His15 phosphoryliert und dieses Phosphat quantitativ auf Glucose übertragen. Andere Proteine können nicht mehr phosphoryliert werden.

Abbildung 4:

Vereinfachte Übersicht über das Katabolitrepressionssystem in *B. subtilis*

Die Abbildungsbeschreibung befindet sich auf der nächsten Seite.

Abbildung 4:

Vereinfachte Übersicht über das Katabolitrepressionssystem in *B. subtilis*

(a): Regulation in Abwesenheit von Glucose. Die Konzentration an HPr-His15-P ist durch die Abwesenheit eines bevorzugten Zuckers hoch, das Phosphat kann somit auf Regulatoren alternativer katabolischer Operons, wie z. B. Antiterminatoren und katabolische Enzyme übertragen werden, wodurch diese Proteine und damit alternative Stoffwechselwege aktiviert werden. EI + EIIA,B,C: Enzyme des Phosphotransferase-Systems.

(b): Regulation in Anwesenheit von Glucose. Das Phosphat von HPr-His15-P wird zunächst nahezu quantitativ auf Glucose nach deren Transport in die Zelle übertragen, weshalb andere Proteine nicht mehr phosphoryliert werden können. Alternative Stoffwechselwege werden dadurch abgeschaltet. (1). Wenn sich Glycolyseintermediate wie Fructose-1,6-Bisphosphat anreichern, wird die Kinase-Aktivität der HPr-Kinase/Phosphatase stimuliert und HPr am Ser46 phosphoryliert, wonach es nicht mehr für die Phosphorylierung der Glucose zur Verfügung steht. HPr-Ser46-P kann nun seinerseits mit CcpA heterodimerisieren und die Transkription an Promotoren mit *cre*-Elementen regulieren. (2). Sinkt die ATP-Konzentration in der Zelle, was mit einer steigenden Phosphat-Konzentration einhergeht, wird die Phosphatase-Aktivität der HPr-Kinase/Phosphatase aktiviert. Dadurch kann HPr nicht mehr mit CcpA interagieren und dieses die *cre*-Elemente nicht mehr binden. Die Phosphorylierungszustände His15-P und Ser46-P schließen sich gegenseitig aus.

konstitutiv exprimierte CcpA in Abhängigkeit vom metabolischen Zustand der Zelle agieren kann. Der CcpA-HPr-Ser46-P-Komplex ist nun in der Lage, jedes der unter *cre*-Element-Kontrolle stehenden Gene zu regulieren. In Abhängigkeit von der Position des *cre*-Elements treten dabei verschiedene Formen der Repression oder auch der Aktivierung auf. *Cre*-Elemente, die sich upstream der RNA-Polymerase-Bindungsstelle befinden, wirken dabei meist aktivierend und erfordern eine direkte Interaktion von CcpA und der RNAP. Beispiele hierfür sind das *ackA*-Gen, das die Acetat-Kinase codiert (Turinsky *et al.*, 1998), das *pta*-Gen, das die Phosphotransacetylase codiert (Shin *et al.*, 1999) oder das *ilvB*-Gen, das die große Untereinheit der Acetolactat-Synthase, welche bei der Biosynthese von Leucin und Valin eine Rolle spielt, codiert (Tojo *et al.*, 2005). Auf die Bedeutung dieser drei Gene wird später noch genauer eingegangen.

Cre-Elemente, die mit der Promotor-Region überlappen, vermitteln in den meisten Fällen durch eine sterische Behinderung der RNAP-Bindung Katabolitrepression, wie z. B. bei den *amyE*-, *bglP*- und *dctP*-Genen, welche verschiedene Abbauenzyme oder Transporter für alternative Nahrungsquellen wie Stärke, Disaccharide oder diverse C_4-Dicarboxylate codieren (Nicholson *et al.*, 1987; Krüger *et al.*, 1996; Asai *et al.*, 2000). Ein häufig beobachteter

Repressionsmechanismus bei *cre*-kontrollierten Promotoren ist die Blockierung der Elongation, welche in der Regel dann auftritt, wenn sich das *cre*-Element downstream des Transkriptionsstartpunktes befindet. Man findet diese Art der Repression z. B. bei der Citrat-Synthase (*citZ*) (Kim *et al.*, 2002), einem Magnesium-abhängigen Citrat-Transporter (*citM*) (Yamamoto *et al.*, 2000) oder einem Transporter für Trehalose (*treP*) (Schöck & Dahl, 1996).

Beim Wachstum in einem Medium, das ausreichende Mengen bevorzugter PTS-Zucker enthält, wird der intrazelluläre Stoffffluss in *B. subtilis* drastisch umgestellt, wie man anhand der durch CcpA aktivierten und reprimierten Gene sehen kann. Unter normalen Wachstumsbedingungen werden die aufgenommenen Kohlenstoffquellen zunächst in die entsprechende Stelle der Glycolyse eingespeist und anschließend im Citrat-Zyklus komplett abgebaut, um eine maximale ATP-Ausbeute pro aufgenommenem Nährstoff-Molekül zu gewährleisten. Sind hingegen ausreichende Mengen bevorzugter PTS-Zucker wie Glucose oder Fructose vorhanden, wird primär Glycolyse betrieben, da diese zwar nicht der effizienteste, aber der schnellste Weg ist, um ATP zu produzieren. Dies führt zu einem starken Anstieg an Pyruvat und Acetyl-CoA in der Zelle, da durch die Inhibierung der Citrat-Synthase durch CcpA das Acetyl-CoA nicht in den Citrat-Zyklus einfließen kann. Die Zelle steht nun vor dem Problem, diese beiden sich anreichernden Metabolite zu entsorgen (Sauer & Eikmanns, 2005). Acetyl-CoA wird dabei durch die beiden von CcpA aktivierten Enzyme Pta und AckA in Acetat umgewandelt, welches anschließend aus der Zelle sezerniert wird und bei Bedarf, z. B. wenn die bevorzugte Zuckerquelle verbraucht sein sollte, wieder aufgenommen werden kann. Ein anderer Teil des Acetyl-CoA wird der Fettsäuresynthese zugeführt, da mit optimalen Nährstoffbedingungen auch meist erhöhte Teilungsraten und so ein großer Bedarf nach Zellmembranbestandteilen einhergehen. Ein Teil des angesammelten Pyruvats wird durch das ebenfalls durch CcpA aktivierte *alsSD*-Operon in Acetoin umgewandelt, welches wie Acetat in das umgebende Medium abgegeben werden kann (Renna *et al.*, 1993). Der andere Teil wird, katalysiert durch die Acetolactat-Synthase über die Zwischenstufe Acetolactat der Synthese verzweigtkettiger Aminosäuren zugeführt, um dem erhöhten Proteinbedarf sich schnell teilender Zellen zu genügen (Shivers & Sonenshein, 2005).

Durch bioinformatische Analyse des *B.-subtilis*-Genoms wurde ein Protein entdeckt, das eine dem HPr ähnliche Sequenz aufweist: Crh (Galinier *et al.*, 1997). Es wurde gezeigt, dass dieses Protein durch die HPr-Kinase/Phosphatase phosphoryliert werden kann und auch mit CcpA interagiert. Eine genaue Funktion dieses Proteins ist noch nicht bekannt, es gibt jedoch Hinweise darauf, dass es die CCR-Funktion von HPr beim Wachstum auf Nicht-

Kohlenhydrat-Substraten wie Succinat oder Glutamat übernimmt (Warner & Lolkema 2003, Görke *et al.*, 2004).

1.2.3. CcpA-unabhängige Katabolitrepression

Obwohl CcpA einen Großteil der unter Katabolitrepression und -aktivierung stehenden Gene der Zelle kontrolliert, zeigten Micro-Array-Analysen, dass es einige Gene gibt, die auch in einem CcpA-Knockout-Stamm oder einem Stamm, in dem HPr nicht an Ser46 phosphoryliert werden kann, durch die Anwesenheit von Glucose im Medium reprimiert werden (Yoshida *et al.*, 2001; Lulko *et al.*, 2007). Dies führte zur Identifizierung weiterer Katabolit-Kontroll-Proteine, die im Folgenden kurz vorgestellt werden sollen:

CcpB, ein zu CcpA paraloges Protein, ist an der Katabolitrepression einiger weniger katabolischer Operons zur Verwertung von Gluconat und Xylose beteiligt und scheint vor allem beim Wachstum auf festen Medien aktiv zu sein, wurde aber seit seiner Entdeckung vor über 10 Jahren nicht weiter erforscht (Chauvaux *et al.*, 1998).

CcpC reprimiert die Gene *citZ*, *citB* und *citC*, die die Enzyme der ersten drei Schritte des Citrat-Zyklus codieren (Jourlin-Castelli *et al.*, 2000). Dabei wird CcpC zum einen durch CcpA und zum anderen durch sich selbst negativ reguliert (Kim *et al.*, 2002; Kim *et al.*, 2003), was die Repressorkonzentration auf ein niedriges, aber für effiziente Repression ausreichendes Niveau reduziert. Citrat wirkt als negativer Regulator von CcpC, sodass der Citrat-Zyklus wieder ablaufen kann, sollte sich Citrat in größeren Mengen in der Zelle ansammeln. Dies und der Wegfall der Repression durch CcpA führen außerdem zu einer Erhöhung der Konzentration an CcpC, sodass die Repression des Citratzyklus bei plötzlicher Citratknappheit sofort wieder einsetzen kann.

CggR (central glycolytic genes repressor) reprimiert die Expression des *cggR-gapA-pgk-tpi-pgm-eno*-Operons, welches den Repressor selbst und alle Glycolyseenzyme, die für den weiteren Abbau der C_3-Intermediate der Glycolyse nötig sind, codiert (Fillinger *et al.*, 2000). Da alle Enzyme außer GapA auch für die Gluconeogenese notwendig sind, besitzt das Operon einen zweiten Promotor, der für eine konstante Menge der verbleibenden Enzyme in der Zelle sorgt (Ludwig *et al.*, 2001). GapA, zusammen mit GapB, stellt eine Besonderheit in *B. subtilis* dar, da beide Enzyme die gleiche Reaktion katalysieren, aber ersteres nur während der Glycolyse, zweites ausschließlich während der Gluconeogenese aktiv ist, während diese Reaktion in den meisten Mikroorganismen nur von einem einzigen Enzym katalysiert wird. Die Affinität von CggR zu seinen Operatoren wird durch Fructose-1,6-Bisphosphat negativ

reguliert (Doan & Aymerich, 2003). Da die Konzentration an Fructose-1,6-Bisphosphat während der Glycolyse deutlich höher als während der Gluconeogenese ist, stellt es ein geeignetes Signal für die Regulation von CggR dar. Wie weiter oben erwähnt, fungiert Fructose-1,6-Bisphosphat auch als Aktivator der Kinase-Aktivität der HPr-Kinase und stellt somit das zentrale Signalmolekül der Katabolitrepression in *B. subtilis* dar.

1.3. Der Transkriptionsfaktor CcpN

Der letzte bisher entdeckte Transkriptionsfaktor, der an der Katabolitrepression beteiligt ist, wurde CcpN (catabolite control protein of gluconeogenic genes) genannt, da er im Zuge der Aufklärung der Regulation der beiden Gene *pckA* (PEP-Carboxykinase) und *gapB* (Glycerinaldehyd-3-Phosphat-Dehydrogenase B), die ausschließlich in der Gluconeogenese aktiv sind, entdeckt wurde (Servant *et al.*, 2005). Zeitgleich dazu wurde CcpN im Rahmen meiner Diplomarbeit unabhängig davon als Transkriptionsregulator einer kleinen RNA, SR1, identifiziert (Licht *et al.*, 2005). SR1 ist ein negativer Regulator von AhrC, das wiederum Gene des Arginin-Abbaus positiv und der Arginin-Biosynthesegene negativ reguliert (Heidrich *et al.*, 2006). Es konnte gezeigt werden, dass SR1 durch Basenpaarung mit der *ahrC*-mRNA deren Struktur ändert und dadurch die Translationsinitiation inhibiert (Heidrich *et al.*, 2007). Für alle drei Gene, *sr1*, *pckA* und *gapB*, konnte eine starke Repression in Anwesenheit einer beliebigen in die Glycolyse einfließenden Zuckerquelle, wie z. B. Glucose, Fructose oder Glycerin gezeigt werden. Servant *et al.* zeigten, dass CcpN für effizientes Wachstum unter glycolytischen Bedingungen essentiell ist und *ccpN*-Knockout-Stämme stark in ihrer Wachstumsgeschwindigkeit limitiert sind. Unter gluconeogenetischen Bedingungen konnte ein schwacher wachstumsbeschleunigender Effekt eines *ccpN*-Knockout-Stammes nachgewiesen werden (Servant *et al.*, 2005).

Das *ccpN*-Gen wird zusammen mit *yqfL* als bicistronische mRNA transkribiert, die konstitutiv exprimiert ist. Homologe von *ccpN* wurden in zahlreichen Firmicuten, wie z. B. *Bacillus halodurans*, *Geobacillus stearothermophilus*, *Bacillus cereus* und *Bacillus anthracis* gefunden. Dem YqfL-Protein konnte bisher noch keine spezifische Funktion zugewiesen werden, es wurde lediglich gezeigt, dass es als positiver Regulator der Expression von *gapB* und *pckA* wirkt, allerdings nicht auf den metabolischen Zustand der Zelle reagiert. Die spezifische Bindung von CcpN an seine Operatoren wurde mittels EMSA und DNase I-Footprints gezeigt. Dabei wurde deutlich, dass CcpN offensichtlich zwei Operatoren am *gapB*- und *sr1*-Promotor und einen ausgedehnten Operator am *pckA*-Promotor besitzt sowie

bei der Bindung an die DNA mehrere DNase-I-hypersensitive Stellen erzeugt, was in der Regel auf eine Änderung der DNA-Struktur hinweist (Servant *et al.*, 2005; Licht *et al.*, 2005). Licht *et al.* konnten jedoch anhand des *sr1*-Promotors zeigen, dass ein Operator ausreichend ist, im CcpN binden zu können. Mit Hilfe von EMSAs mit mutierten Oligonucleotiden konnte folgende provisorische Konsensussequenz für CcpN abgeleitet werden: DDDTGTGYYATACTRDK. Eine Suche nach dieser Sequenz im *B. subtilis*-Genom zeigte, dass zahlreiche Gene einen CcpN-Operator in der Promotorregion besaßen, darunter auch *pckA* und *gapB*. Allerdings wiesen nur diese beiden neben dem *sr1*-Operator Bindungsaktivität auf.

Inzwischen wurden auch die biophysikalischen Eigenschaften von CcpN im Detail untersucht. Dabei wurde festgestellt, dass CcpN in der Zelle als Dimer vorliegt (Zorrilla *et al.*, 2008). Die erhaltenen Informationen bezüglich der Bindungsstöchiometrie von CcpN weisen allerdings Widersprüche zu bereits vorhandenen Daten auf. So wurde durch „fluorescence-cross-correlation-spectroscopy“ eine Bindungsstöchiometrie von zwei Molekülen CcpN pro *gapB*-Operator und 4 Molekülen pro *pckA*-Operator gefunden, was allerdings den Beobachtungen im EMSA widerspricht, der eine identische Größe der Protein-DNA-Komplexe an allen Operatoren zeigte. Dieser offensichtliche Widerspruch wurde allerdings von den Autoren nicht diskutiert.

Wesentlich besser hingegen ist die Steuerung der intrazellulären Metabolismus-Flüsse, die von CcpN reguliert werden, erforscht (Tännler *et al.*, 2008). Durch die Messung der Konzentration verschiedener Metabolite in Wildtyp- und *ccpN*-Knockout-Stämmen wurde festgestellt, dass ein *ccpN*-Knockout unter glycolytischen Bedingungen durch die unkontrollierte Expression von *pckA* erhebliche Mengen Energie durch einen so genannten „futile cycle“ verbraucht. Dabei wird PEP durch die Pyruvat-Kinase in Pyruvat umgewandelt, das wiederum unter ATP-Verbrauch durch die Pyruvat-Carboxylase in Oxalacetat metabolisiert wird. Durch die hohe Aktivität von PckA wird das Oxalacetat unter erneutem ATP-Verbrauch wieder in PEP überführt. Dieser Zyklus läuft dann ungehemmt ab, verbraucht ständig ATP und entzieht dem Citratzyklus zudem große Mengen Oxalacetat. Tatsächlich ist der Mangel an Oxalacetat, der durch eine unregulierte PckA-Aktivität hervorgerufen wird, die Hauptursache für den Wachstumsdefekt, den ein *ccpN*-Knockout-Stamm zeigt, da durch den geringen Oxalacetat-Pool die Aspartat-Synthese nicht mehr ablaufen kann. Entsprechend kann ein *ccpN*-Knockout-Stamm unter glycolytischen Bedingungen eine dem Wildtyp gleichende Wachstumsrate erreichen, sofern er auf Aspartat angezogen wird. Neben „futile cycling“ und Oxalacetat-Mangel ist ein *ccpN*-Knockout-Stamm auch durch einen drastisch

erhöhten Metabolit-Fluss durch den Pentosephosphat-Weg gekennzeichnet. Für diesen Effekt ist die Überexpression von *gapB* verantwortlich, welches einen Großteil des gebildeten 1,3-Bisphospho-Glycerats sofort wieder in Glycerinaldehyd-3-Phosphat umwandelt. Dadurch entsteht ein „Metabolit-Stau“ im oberen Bereich der Glycolyse, der von der Zelle dadurch abgefangen wird, dass größere Mengen Fructose-1,6-Bisphosphat in den Pentosephosphat-Weg geleitet werden.

1.4. Zielsetzung

Bakterien müssen sich ständig auf sich wechselnde Umweltbedingungen, die unter anderem durch das Nahrungsangebot, aber auch durch Änderungen in der Temperatur oder der Osmolarität gekennzeichnet sind, einstellen. Die Anpassung an verschiedene Nahrungsquellen geschieht dabei durch den Prozess der Katabolitrepression. Dieser Vorgang ist in *B. subtilis* trotz über zwanzigjähriger Forschung noch immer nicht ganz verstanden. Die Entdeckung und Charakterisierung neuer daran beteiligter Faktoren, wie z. B. CcpN oder das Crh-Protein helfen stetig dabei, das Bild zu vervollständigen. Im Rahmen dieser Arbeit soll der letzte entdeckte an der Katabolitrepression beteiligte Transkriptionsfaktor CcpN näher charakterisiert werden, um neue Erkenntnisse bezüglich seiner DNA-Bindungseigenschaften, seiner Regulation und seines Wirkungsmechanismus zu gewinnen.

Der Transkriptionsrepressor CcpN wurde in den beiden Arbeiten, die seine Entdeckung beschreiben, nur ansatzweise hinsichtlich seiner DNA-Bindungseigenschaften untersucht. Da die erhaltenen DNase I-Footprints nur eine sehr ungenaue Eingrenzung des Bindungsbereichs ermöglichen, sollte in dieser Arbeit mittels verschiedener Interferenz-Footprint-Methoden eine basengenaue Auflösung der Protein-DNA-Kontakte an den CcpN-Operatoren erhalten werden. Dabei galt es zu klären, ob auch der *pckA*-Operator wie der *sr1*- und der *gapB*-Operator aus zwei Bindungsstellen besteht und wie die Kontaktverteilung an den beiden Bindungsstellen eines Promotors, die untereinander nur äußerst geringe Sequenzhomologien aufweisen, aussieht. Weiterhin sollte untersucht werden, ob CcpN die beiden Einzeloperatoren eines Promotors kooperativ oder unabhängig voneinander bindet. Die Bindung eines Proteins an die DNA kann entweder enthalpisch oder entropisch angetrieben werden. Die Untersuchung der CcpN-DNA-Interaktion bei verschiedenen Temperaturen sollte Aufschluss über die Verhältnisse im Falle von CcpN geben (**Manuskript I**).

Eine erste Charakterisierung von CcpN zeigte, dass seine Expression nicht reguliert ist. Da aber alle Transkriptionsfaktoren, um ihre Aufgabe erfüllen zu können, einer Regulation bedürfen, sollte in dieser Arbeit nach dem Regulator, der die CcpN-Funktion moduliert, gesucht werden. Dazu sollte zunächst die Expression einer p_{sr1}-*lacZ*-Transkriptionsfusion in verschiedenen *B.-subtilis*-Stämmen, die Defekte an definierten Stellen der Glycolyse aufweisen, untersucht werden, um einen Anhaltspunkt über einen möglichen Metabolit-Regulator zu erhalten. Danach sollten ein *in vitro*-Transkriptionssystem für CcpN etabliert werden, um verschiedene intrazelluläre Metabolite auf ihre Fähigkeit zu untersuchen, den Repressionseffekt von CcpN zu beeinflussen. Sollte ein potentieller Regulator gefunden werden, sollte dessen Interaktion mit CcpN mit Hilfe von Circular-Dichroismus-Messungen (CD-Messungen) bestätigt werden. Letztendlich sollte untersucht werden, ob ein identifizierter Ligand von CcpN in der Lage ist, das DNA-Bindungsverhalten von CcpN zu verändern und damit einen Hinweis bezüglich des Repressionsmechanismus von CcpN liefern (**Manuskript II**).

Transkriptionsrepressoren können auf viele verschiedene Weisen wirken, abhängig vom Repressor selbst, aber auch von der Position der Operatoren in Bezug auf den Promotor. Da im Falle von CcpN die Operator-Position an den einzelnen Promotoren unterschiedlich ist, sollte untersucht werden, ob CcpN an den verschiedenen Operatoren unterschiedliche Repressionsmechanismen nutzt. Dazu sollte der Einfluss von CcpN auf die einzelnen Schritte der Transkriptionsinitiation untersucht werden. Mit Hilfe von EMSAs sollte die Bildung des geschlossenen Komplexes untersucht werden, anschließend sollte die Bildung offener Komplexe an den drei Operatoren in An- und Abwesenheit von CcpN analysiert werden. Mit Hilfe von *in vitro*-Transkriptions-Versuchen sollte ein Einfluss von CcpN auf die Bildung von abortiven Transkripten und damit des Initiationskomplexes und des Elongationskomplexes festgestellt werden. Da sich die Operatoren von CcpN in unmittelbarer räumlicher Nähe zur Bindungsstelle für die RNA-Polymerase befinden, sollte mit Hilfe von Far-Western-Blots und Co-Elutions-Versuchen eine mögliche Interaktion zwischen CcpN und Untereinheiten der RNA-Polymerase nachgewiesen werden (**Manuskript III**).

Bereits bei der ersten Charakterisierung von CcpN gab es Hinweise, dass die intrazelluläre Konzentration dieses Repressors verhältnismäßig hoch sein muss. Im dies zu verifizieren, sollte die Konzentration von CcpN in der Zelle bestimmt werden. Im Anschluss daran sollte mit Hilfe von bioinformatischen und molekularbiologischen Mitteln nach weiteren CcpN-

regulierten Genen im Genom von *B. subtilis* gesucht werden. Dazu sollten potentielle CcpN-Operatoren in EMSAs auf ihre Proteinbindungsfähigkeit untersucht und anschließend mittels DNase I- und Interferenz-Footprints genauer untersucht werden, um Gemeinsamkeiten mit und Unterschiede zu den bereits bekannten Promotoren festzustellen. Positive Kandidaten sollten anschließend mit Hilfe von *lacZ*-Transkriptionsfusionen auf ihre Funktionalität *in vivo* untersucht und die Ergebnisse bei Bedarf durch *in vitro*-Transkriptions-Versuche untermauert werden (**Manuskript IV**).

2. Übersicht zu den Manuskripten

Manuskript I

Andreas Licht & Sabine Brantl

Transcriptional repressor CcpN from *Bacillus subtilis* compensates asymmetric contact distribution by cooperative binding.

Journal of Molecular Biology, 364: 434-448 (2006)

In dieser Publikation werden die Sequenzanforderungen für eine Interaktion des Transkriptionsfaktors CcpN aus *B. subtilis* mit seinen Operatoren am *sr1*-, *pckA*- und *gapB*-Promotor mittels Interferenz-Footprinting untersucht. Außerdem wird mit Hilfe von DNase I-Footprints gezeigt, dass CcpN seine Operatoren kooperativ bindet und es wurde ermittelt, dass die CcpN-Operator-Interaktion enthalpisch getrieben ist.

Alle Experimente in dieser Publikation wurden von Andreas Licht erdacht, durchgeführt und ausgewertet. Das Manuskript wurde von Andreas Licht verfasst. Sabine Brantl und Andreas Licht haben das Manuskript gemeinsam korrigiert und verbessert.

Manuskript II

Andreas Licht, Ralph Golbik & Sabine Brantl

Identification of ligands affecting the activity of the transcriptional repressor CcpN from *Bacillus subtilis*.

Journal of Molecular Biology, 380: 17-30 (2008)

Diese Publikation beschreibt die Suche nach intrazellulären Regulatoren des Transkriptionsrepressors CcpN aus *B. subtilis*. Mit Hilfe von *in vitro*-Transkriptions-Versuchen wurden ATP und niedriger pH-Wert als Aktivatoren und ADP als Repressor der Repressionsaktiviät von CcpN identifiziert. Ferner wurde mit Hilfe von CD-Messungen eine Änderung der Struktur von CcpN bei ATP-Bindung im Sauren festgestellt und mit Hilfe von EMSAs gezeigt, dass die DNA-Bindungsaktivität von CcpN durch seine Regulatoren nicht beeinflusst wird.

Alle Experimente in dieser Publikation wurden von Andreas Licht erdacht und durchgeführt. Ralph Golbik führte die Reinigung von CcpN mittels FPLC durch, vermittelte

die Grundkenntnisse in der Bedienung des CD-Spektrometers und wertete die CD-Messungen aus. Alle anderen Versuche wurden von Andreas Licht ausgewertet. Das Manuskript wurde von Andreas Licht verfasst. Sabine Brantl, Ralph Golbik und Andreas Licht haben das Manuskript gemeinsam korrigiert und verbessert.

Manuskript III

Andreas Licht & Sabine Brantl

The transcriptional repressor CcpN from *Bacillus subtilis* uses different repression mechanisms at different promoters.

Revision eingereicht bei *Journal of Biological Chemistry* am 03.08.2009

Die Aufklärung des Repressionsmechanismus des Transkriptionsfaktors CcpN aus *B. subtilis* wird in dieser Publikation beschrieben. Mittels EMSA, „open complex formation"-Assays und *in vitro*-Transkription konnte gezeigt werden, dass CcpN am *gapB*-Promotor die die abortive Transkription inhibiert, während es am *pckA*- und *sr1*-Promotor die Bildung eines Elongationskomplexes verhindert. Weiterhin wurde eine spezifische Interaktion von CcpN und der α-Untereinheit der RNA-Polymerase detektiert, welche mit hoher Wahrscheinlichkeit für die Repression am *sr1*- und *pckA*-Promotor verantwortlich ist.

Alle Experimente in dieser Publikation wurden von Andreas Licht erdacht, durchgeführt und ausgewertet. Das Manuskript wurde von Andreas Licht verfasst. Sabine Brantl und Andreas Licht haben das Manuskript gemeinsam korrigiert und verbessert.

Manuskript IV

Rita A. Eckart, Sabine Brantl & Andreas Licht

Search for additional targets of the transcriptional regulator CcpN from *Bacillus subtilis*.

Akzeptiert bei *FEMS Microbiology Letters* am 31.07.2009

In dieser Arbeit wird die Suche nach neuen Zielgenen, die unter der Kontrolle des Transkriptionsrepressors CcpN aus *B. subtilis* stehen, beschrieben. Dabei wurde mit einer Kombination aus bioinformatischer Suche, *in vitro*- und *in vivo*-Experimenten *thyB* (Thymidylat-Synthase B) als neues Zielgen identifiziert, an welchem CcpN, im Gegensatz zu den bisher bekannten Zielgenen, als Aktivator wirken kann. Eine Suche in nahe verwandten

Bakterienspezies zeigte außerdem, dass auch in diesen eine CcpN-vermittelte Regulation von *thyB* wahrscheinlich ist.

Alle Experimente dieser Publikation wurden von Andreas Licht erdacht. Andreas Licht führte die bioinformatische Suche, den DNase-I-Footprint des mutierten *thyB*-Promotors, die *lacZ*-Aktivitätsbestimmung am mutierten *thyB*-Promotor und die *in vitro*-Transkription durch und wertete diese aus. Alle anderen Versuche wurden von Rita Eckart durchgeführt und ausgewertet. Das Manuskript wurde von Andreas Licht verfasst. Sabine Brantl, Rita Eckart und Andreas Licht haben das Manuskript gemeinsam korrigiert und verbessert.

3. Transcriptional repressor CcpN from *Bacillus subtilis* compensates asymmetric contact distribution by cooperative binding

(Manuskript I)

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Transcriptional Repressor CcpN from *Bacillus subtilis* Compensates Asymmetric Contact Distribution by Cooperative Binding

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Carbon catabolite repression in *Bacillus subtilis* is carried out mainly by the major regulator CcpA. In contrast, sugar-dependent repression of three genes, *sr1* encoding a small untranslated RNA, and two genes, *gapB* and *pckA*, coding for gluconeogenic enzymes is mediated by the recently identified transcriptional repressor CcpN. Since previous DNase I footprinting yielded only basic information on the operator sequences of CcpN, chemical interference footprinting studies were performed for a precise contact mapping. Methylation interference, potassium permanganate and hydroxylamine footprinting were used to identify all contacted residues in both strands in the three operator sequences. Furthermore, ethylation interference experiments were performed to identify phosphate residues essential for CcpN binding. Here, we show that each operator has two binding sites for CcpN, one of which was always contacted more strongly than the other. The three sites that exhibited close contacts were very similar in sequence, with only a few slight variations, whereas the other three corresponding sites showed several deviations. Gel retardation assays with purified CcpN demonstrated that the differences in contact number and strength correlated well with significantly different K_D values for the corresponding single binding sites. However, quantitative DNase I footprinting of whole operator sequences revealed cooperative binding of CcpN that, apparently, compensated the asymmetric contact distribution. Based on these data, possible consequences for the repression mechanism of CcpN are discussed.

Keywords: CcpN; transcriptional repressor; chemical footprinting; carbon catabolite repression; DNA-protein-interaction

Introduction

Although many bacteria, including *Bacillus subtilis*, are able to utilise a vast number of other nutrients,[1,2] glucose is their preferred carbon source.[3] Therefore, cells need to shut-down other catabolic pathways in the presence of glucose to maximise the energy yield.[4] This is accomplished by so-called catabolite repression. In *Escherichia coli*, catabolite repression is mediated by the central signalling molecule cAMP and its receptor protein CRP.[5,6] By contrast, *B. subtilis* does not encode a CRP homologue nor does it produce detectable amounts of cAMP under aerobic conditions.[7] Instead, catabolite repression in *B. subtilis* is carried out mainly by the concerted action of CcpA and HPr-Ser46-P, which can interact to form a transcriptional repressor or activator, regulating genes involved in carbon catabolism.[8] However, it has been shown recently that at least two genes, *gapB* and *pckA*, are down-regulated in the presence of glucose, independent of CcpA.[9,10] Instead, they are regulated by a novel transcriptional repressor found by transposon mutagenesis screening for derepression of *gapB* and, therefore, named CcpN (for control catabolite protein of gluconeogenic genes).[11] The *gapB* gene encodes the rare isotype B of glyceraldehyde-3-phosphate dehydrogenase, and its gene product catalyses the conversion of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate, but only during gluconeogenesis.[9] The *pckA* gene codes for another enzyme required for the synthesis of glucose from

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Krebs cycle intermediates, PEP carboxykinase, which catalyses the conversion of oxaloacetate to phosphoenolpyruvate.[12]

The *ccpN* gene is cotranscribed with the *yqfL* gene, resulting in a bicistronic mRNA. It was shown that this operon is not autoregulated, but constitutively expressed under both glycolytic and gluconeogenic conditions.[11] Homologues of CcpN have been found in the genomes of other Bacilli, e.g. *B. halodurans*, *B. cereus*, *B. anthracis* and *Geobacillus stearothermophilus*, and in different Firmicutes.[11]

Recently, a third gene regulated by CcpN, *sr1*, has been discovered. This gene codes for a small untranslated RNA, SR1, which has been identified by a systematic search for small RNAs within intergenic regions of the *B. subtilis* genome.[13] *sr1* was expressed during gluconeogenesis, but repressed under glycolytic conditions. The *trans*-acting factor responsible for sugar-mediated repression was identified as CcpN.[13] Previous DNase I footprinting experiments for all three known CcpN operators indicated different locations of the binding regions relative to the transcription start site.

The aim of the present work was to investigate the interaction between CcpN and its operator regions in more detail using chemical interference footprinting. These experiments showed that contact strength varied greatly, depending on the sequence of a given site. Gel retardation assays with single binding sites confirmed these observations. However, quantitative DNase I footprinting experiments with DNA fragments of all three genes spanning the corresponding complete operator sequences indicated cooperative binding of CcpN. The possible impact of these results on the repression mechanism is discussed.

Results

Chemical interference footprinting experiments were performed with CcpN-His_5 (containing five additional C-terminal histidine residues) purified from an *E. coli* over-expression strain. Electrophoretic mobility shift assays (EMSAs) have verified that His-tagged CcpN shows the same binding properties as wild-type CcpN and Northern blots showed that it can exert the function of wild-type CcpN in a *ccpN* knockout strain (data not shown). All nucleotide numbers in the following paragraphs refer to the transcription start sites. The coding strand is always termed the top strand, and the non-coding strand is always termed the bottom strand.

Methylation interference

Methylation interference experiments were performed to determine guanine and adenine bases contacted by CcpN. DNA fragments were modified at purine residues by dimethyl sulphate before CcpN binding. Adenine is methylated at position N3 in the minor groove and guanine is methylated at position N7 in the major groove. Figure 1 shows the positions of the methyl groups interfering with CcpN binding. The top strand of the *sr1* operator exhibited interference at positions $G_{(-53)}$ and $G_{(-51)}$ in site I and, to a weaker extent, at $G_{(-21)}$ and $G_{(-19)}$ in site II. Adenine residues with a major contribution to CcpN binding were found only in site I at the top strand ($A_{(-48)}$ and $A_{(-46)}$), whereas in site II only less close contacts were detected. At the bottom strand, methylation of $G_{(-45)}$ in site I and $G_{(-17)}$ in site II interfered with CcpN binding. Only less close contacts to adenine residues have been found in the bottom strand: $A_{(-52)}$ and $A_{(-47)}$ in site I and $A_{(-20)}$ in site II were contacted by CcpN.

Since the contacts to guanine residues were in all cases closer than those to adenine, these results indicate that CcpN contacts the DNA mainly via the major groove with some auxiliary contacts in the minor groove. Furthermore, the contacts in site II were generally less close than those in site I.

In the *pckA* operator, only three contacts to guanine residues have been observed: $G_{(-38)}$ in site I and $G_{(-15)}$ in site II at the top strand as well as $G_{(-9)}$ in site II at the bottom strand. Binding site II was found to be contacted much more strongly than site I. The same was true for contacts to adenine. Whereas there were some significant contacts in binding site II ($A_{(-17)}$ and $A_{(-12)}$ at the top strand and $A_{(-11)}$ at the bottom strand), only one contacted adenine was detected in site I ($A_{(-36)}$ at the top strand). No significant contact was found in binding site I on the bottom strand.

The *gapB* operator showed a similarly asymmetric contact distribution, but here, contacts were concentrated in binding site I: Close contacts to guanine ($G_{(-17)}$ and $G_{(-15)}$ at the top strand and $G_{(-9)}$ on the bottom strand) were found, whereas in site II only one less closely contacted guanine ($G_{(+22)}$ on the bottom strand) was detected. The same contact distribution was found for adenine residues. Close and medium contacts were observed only in binding site I ($A_{(-19)}$ at the top strand and $A_{(-11)}$, $A_{(-8)}$ at the bottom strand). In binding site II, no close contact to adenine was observed.

Interestingly, contacts in the *sr1* operator were concentrated upstream of the −35 region and, to a lesser extent, in the spacer between −35 and −10, whereas almost no contact was observed directly within the −35 and −10 regions of p_{SR1}. In contrast, in both the *pckA* and *gapB* operator, close contacts were found only within the −10 region, whereas weak binding sites covered the −35 region and the region downstream from the transcription start site in the *pckA* and *gapB* operator, respectively. Moreover, the interference footprinting revealed that each of the three operators had two CcpN binding sites, although they appeared, due to the short spacer region, as one extended site in the previous DNase I footprints of the *pckA* operator.[11]

Potassium permanganate footprinting

Potassium permanganate footprinting was performed to determine contacts of CcpN to thymine

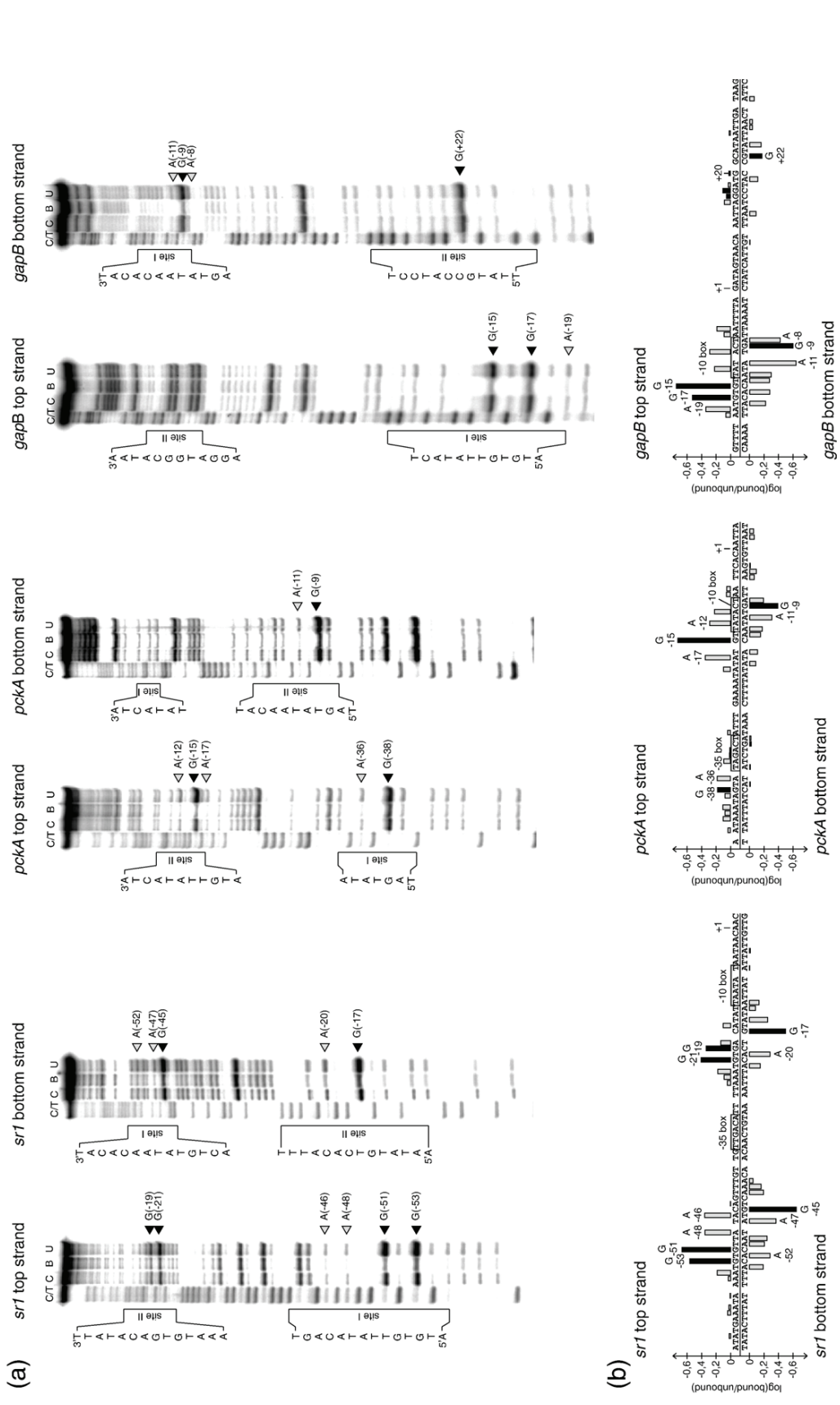

Figure 1. Methylation interference of the *sr1*, *pckA*, and *gapB* operators. (a) C/T, Maxam Gilbert C+T sequencing reaction; C, control (protein-free methylated DNA, this lane is equivalent to a Maxam–Gilbert G>A sequencing reaction); B and U, bound and unbound fraction of methylated DNA subjected to binding with CcpN-His$_6$. The numbers in the gels and column diagrams show the positions of the corresponding nucleotides relative to the transcription start site. Binding sites I and II for CcpN have been denoted according to interference footprinting experiments. Close contacts are indicated by black and grey triangles for G and A, respectively. (b) Column diagrams indicating the relative strength of interference signals for both strands of the three operators. Only positive signals, i.e. signals that indicate contacts, are shown. Measured values are averaged from four independent experiments.

residues within the three operators. $KMnO_4$, a strong oxidising agent, specifically oxidises thymine, thus impeding protein contacts. In addition to thymine, guanine is modified by $KMnO_4$, which results in bands for guanine residues in the gels. Figure 2 shows the positions of the modified thymine interfering with CcpN binding. In general, contact distribution correlated well with that found by methylation interference footprinting. The *sr1* operator exhibited the following strong interference signals in binding site I: $T_{(-52)}$ and $T_{(-50)}$ at the top strand and $T_{(-48)}$, $T_{(-46)}$, $T_{(-44)}$ at the bottom strand. However, in contrast to the contacts to guanine and adenine, contacts to thymine ($T_{(-22)}$ and $T_{(-20)}$ at the top strand) were slightly closer in binding site II. Significant contacts in binding site II have not been found on the bottom strand.

Both in the *pckA* and *gapB* operators, the positions of contacted thymine corresponded perfectly to those identified for guanine and adenine by methylation interference, too. The focus of contacts was in site II in the case of *pckA* (five close contacts, see Figure 2, at the top strand and $T_{(-12)}$ and $T_{(-10)}$ at the bottom strand), while only less close contacts were found in binding site I at the top strand and no significant contact at the bottom strand. The *gapB* operator exhibited strong interference signals only in site I (mainly $T_{(-16)}$ and $T_{(-14)}$ on the top strand and $T_{(-12)}$ and $T_{(-10)}$ on the bottom strand), whereas only less close contacts were found in binding site II at the top strand and no significant contact at the bottom strand. In all three operators, thymine bases that showed the strongest interference signals were located next to contacted guanine bases, together forming the contact center within each binding site.

Hydroxylamine footprinting

NH_2OH footprinting was used to analyse CcpN contacts to cytosine in the three operators. Hydroxylamine, a strong reductive agent, causes ring opening specifically at cytosine bases and, in this way, interferes with contact formation between protein and DNA. Figure 3 shows that contacts to cytosine bases were found in all three operators; however, the contacts were less close compared to the three other bases. Interference signals of almost equal intensity were found in the *sr1* operator in site I ($C_{(-45)}$ at the top strand and $C_{(-53)}$ and $C_{(-51)}$ on the bottom strand) and in site II ($C_{(-17)}$ at the top strand and $C_{(-21)}$ and $C_{(-19)}$ on the bottom strand). The *pckA* operator showed only two contacted cytosine bases in binding site II and no contact in binding site I. Interestingly, in the *gapB* operator, three contacted

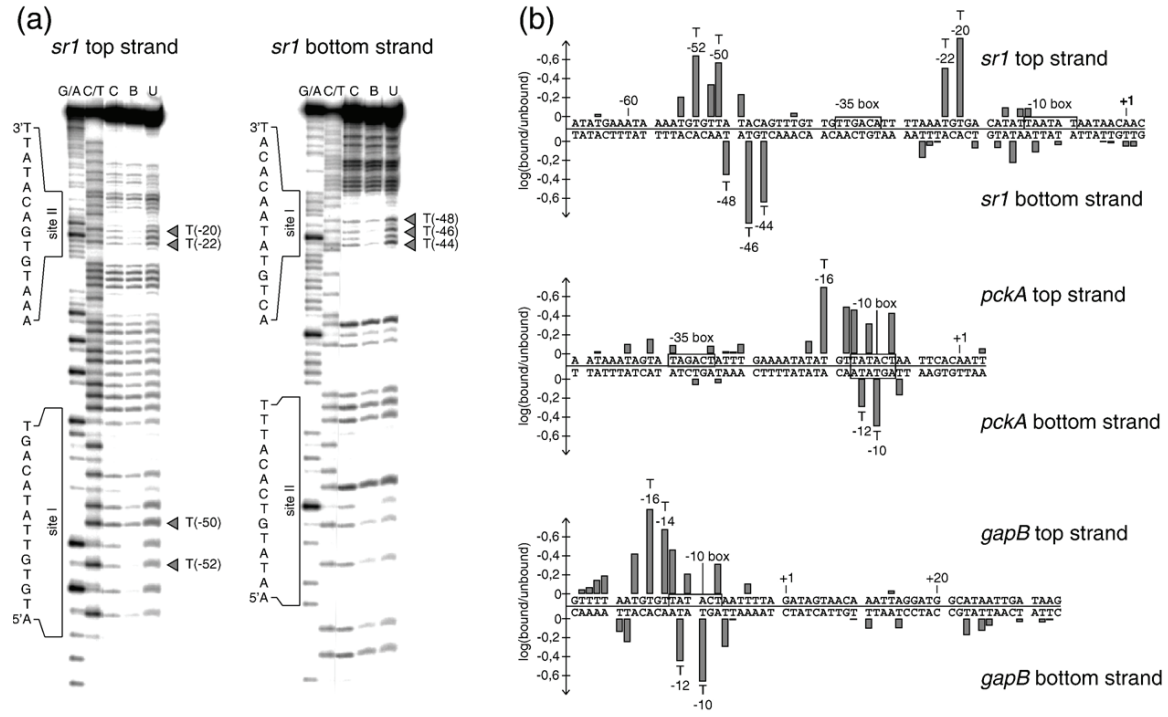

Figure 2. $KMnO_4$ interference of the *sr1*, *pckA* and *gapB* operators. (a) G/A, Maxam–Gilbert G>A sequencing reaction; C/T, Maxam–Gilbert C+T sequencing reaction; C, control (protein-free $KMnO_4$-treated DNA); B and U, bound and unbound fraction of methylated DNA subjected to binding with CcpN-His_5. The numbers in the gels and column diagrams show the positions of the corresponding nucleotides relative to the transcription start site. Binding sites I and II for CcpN are designated as in Figure 1. Close contacts are indicated by dark grey triangles. Only the gels for top and bottom strand of the *sr1* operator are shown. (b) The column diagrams present the relative strength of interference signals for both strands of the three operators. Only positive signals, i.e. signals that indicate contacts, are shown. Measured values are averaged from four independent experiments.

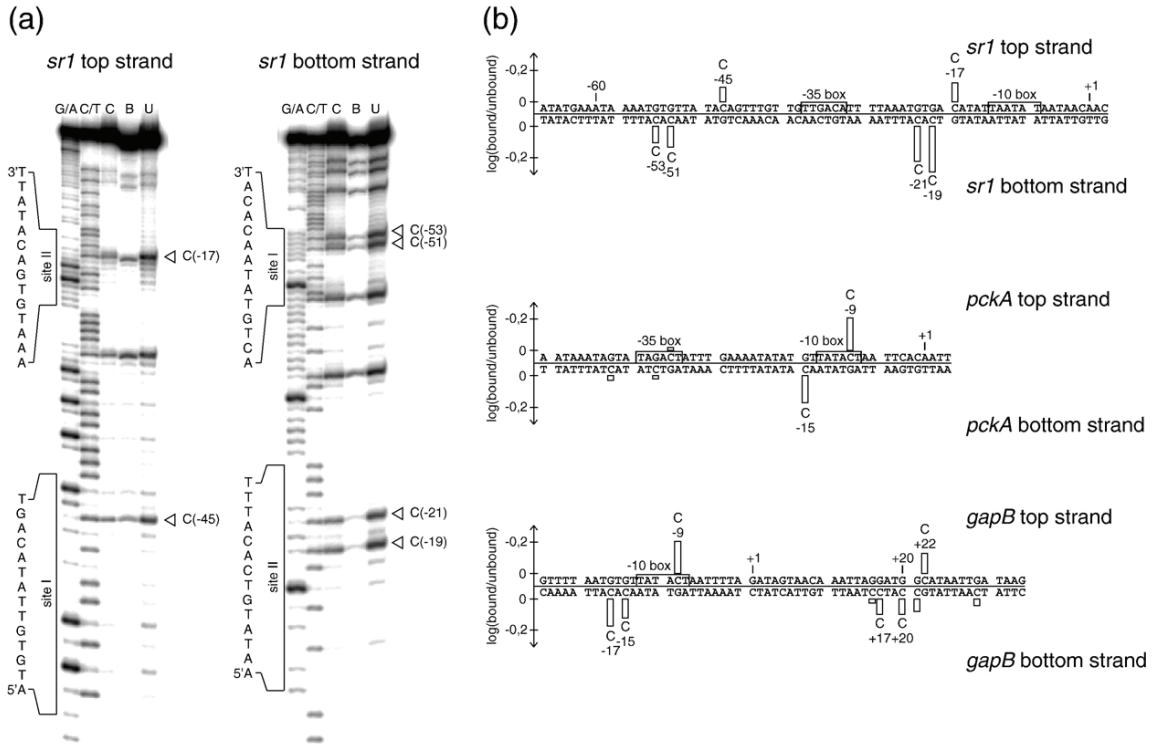

Figure 3. NH_2OH interference of the *sr1*, *pckA* and *gapB* operators (a) G/A, Maxam–Gilbert G>A sequencing reaction; C/T, Maxam–Gilbert C+T sequencing reaction; C, control (protein free NH_2OH-treated DNA); B and U, bound and unbound fraction of methylated DNA subjected to binding with CcpN-His_5. The numbers in the gels and column diagrams show the positions of the corresponding nucleotides relative to the transcription start site. Binding sites for CcpN are designated as in Figure 1. Close contacts are indicated by white triangles. Only the gels for top and bottom strand of the *sr1* operator are shown. (b) The column diagrams present the relative strength of interference signals for both strands of the three operators. As above, only positive signals are shown. Measured values are averaged from four independent experiments.

cytosine bases were found in both site I ($C_{(-9)}$ at the top strand and $C_{(-15)}$ and $C_{(-17)}$ at the bottom strand) and site II ($C_{(+22)}$ at the top strand and $C_{(+17)}$ and $C_{(+20)}$ at the bottom strand). However, due to the weak nature of these interference signals, contacts to cytosine do not seem to play an important role in the CcpN–DNA interaction.

Ethylation interference footprinting

To determine phosphate groups of the DNA backbone contacted by CcpN, ethylation interference experiments were carried out. Figure 4 presents the positions at which ethylation interfered with CcpN binding. Both binding sites in the *sr1* operator showed only two interference signals: In site I, $T_{(-50)}$ at the top strand and $A_{(-47)}$ at the bottom strand were contacted, and in site II, $A_{(-12)}$ and $A_{(-15)}$ at the bottom strand were contacted. In the *pckA* operator, contacts to the sugar-phosphate backbone were detected only in binding site II. Here, $T_{(-14)}$ at the top strand and $A_{(-8)}$ and $T_{(-10)}$ at the bottom strand exhibited interference signals. The same was found for the *gapB* operator, where only binding site I showed two contacts, to $T_{(-14)}$ at the top strand and $T_{(-10)}$ at the bottom strand.

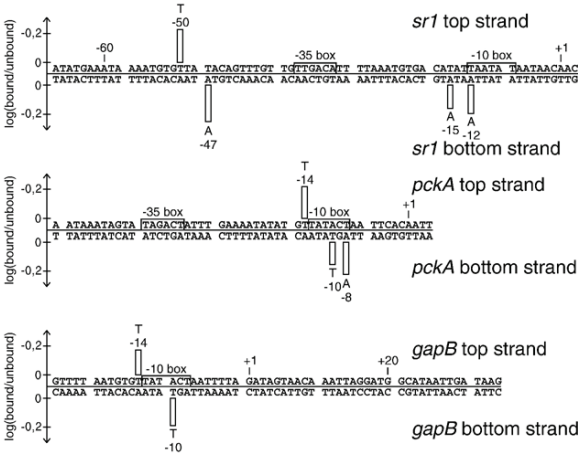

Figure 4. Ethylation interference of the *sr1*, *pckA* and *gapB* operators. The column diagrams present the relative strength of interference signals for both strands of the three operators. Only positive signals, i.e. signals that indicate contacts, are shown. Numbers in the column diagrams designate the positions of the corresponding nucleotides relative to the transcription start site. Measured values are averaged from three independent experiments.

Interestingly, the few DNA-backbone contacts were observed in most cases next to a contacted guanine residue. Obviously, these contacts play only a minor role in the binding of CcpN to its operators. Figure 5 summarises all probed contacts for the three operators.

EMSA

To determine the apparent equilibrium dissociation constants K_D for the CcpN-DNA complex, 23 bp double-stranded oligonucleotides containing a single CcpN binding site were incubated with increasing concentrations of CcpN-His_5 (Figure 6(a)). K_D values were estimated by non-linear regression using the average data from three independent experiments as described in Materials and Methods. The calculated K_D values as well as the binding energy ΔG for the CcpN–DNA interaction for the single sites are summarised in Table 1. Binding energy was calculated with the help of Van't Hoff's reaction isobare $\Delta G = -RT \ln(K)$, where R is the universal gas constant, T is the absolute temperature (in Kelvin) and K is the determined equilibrium association constant. The calculated K_D values for the single binding sites corresponded very well to the contacts that were observed by interference footprinting: Binding site I of the *gapB* operator, the one with the most and closest contacts (see Figure 1), showed the lowest K_D value, indicating a tight protein–DNA interaction, whereas binding site II of *gapB* or site I of *pckA*, both with less close contacts, exhibited high K_D values. Determined dissociation constants ranged from as low as 98 nM (*gapB*, site I) till 4.4 μM (*gapB*, site II).

To test whether the equilibrium dissociation constants differ when using whole operators, double-stranded oligonucleotides containing both CcpN binding sites were incubated with increasing concentrations of CcpN-His_5 (Figure 6(b)). K_D values were estimated by non-linear regression using the average data from three independent experiments as described in Materials and Methods. The apparent equilibrium dissociation constants were determined to be 19.3 nM, 15.5 nM and 12.8 nM for the *sr1*, *pckA* and *gapB* operator, respectively, and correspond well to the values determined by Servant *et al.*[11] All operators showed significantly lower K_D values than the single sites alone. At the *sr1* operator, the average K_D was decreased 30-fold, while the K_D of site I of the *pckA* and site II of the *gapB* operators was decreased 160-fold and 340-fold, respectively.

Figure 5. Overview of the contacts in all three operators. (a) Overview of all contacts. Symbols used to indicate contacts to the bases are shown in the box below. Filled symbols denote close contacts (50%–100% compared to the strongest signal), open symbols represent medium or less close contacts (15%–50% compared to the strongest signal). For clarity, contacts with less than 15% relative strength are not shown. The −35 and −10 regions are boxed, and the transcription start site is indicated. Binding sites I and II are designated on the basis of all interference footprinting experiments. (b) Alignment of the core sequences of all binding sites. Positions that coincide with the consensus are shown in bold.

Quantitative DNase I footprinting

Since occupancy of single sites is not detectable in EMSA, the affinity of CcpN to the single sites within the complete operator was measured by quantitative DNase I footprinting. This technique allows us to determine K_D values for site I and site II separately, even if they are located on one DNA

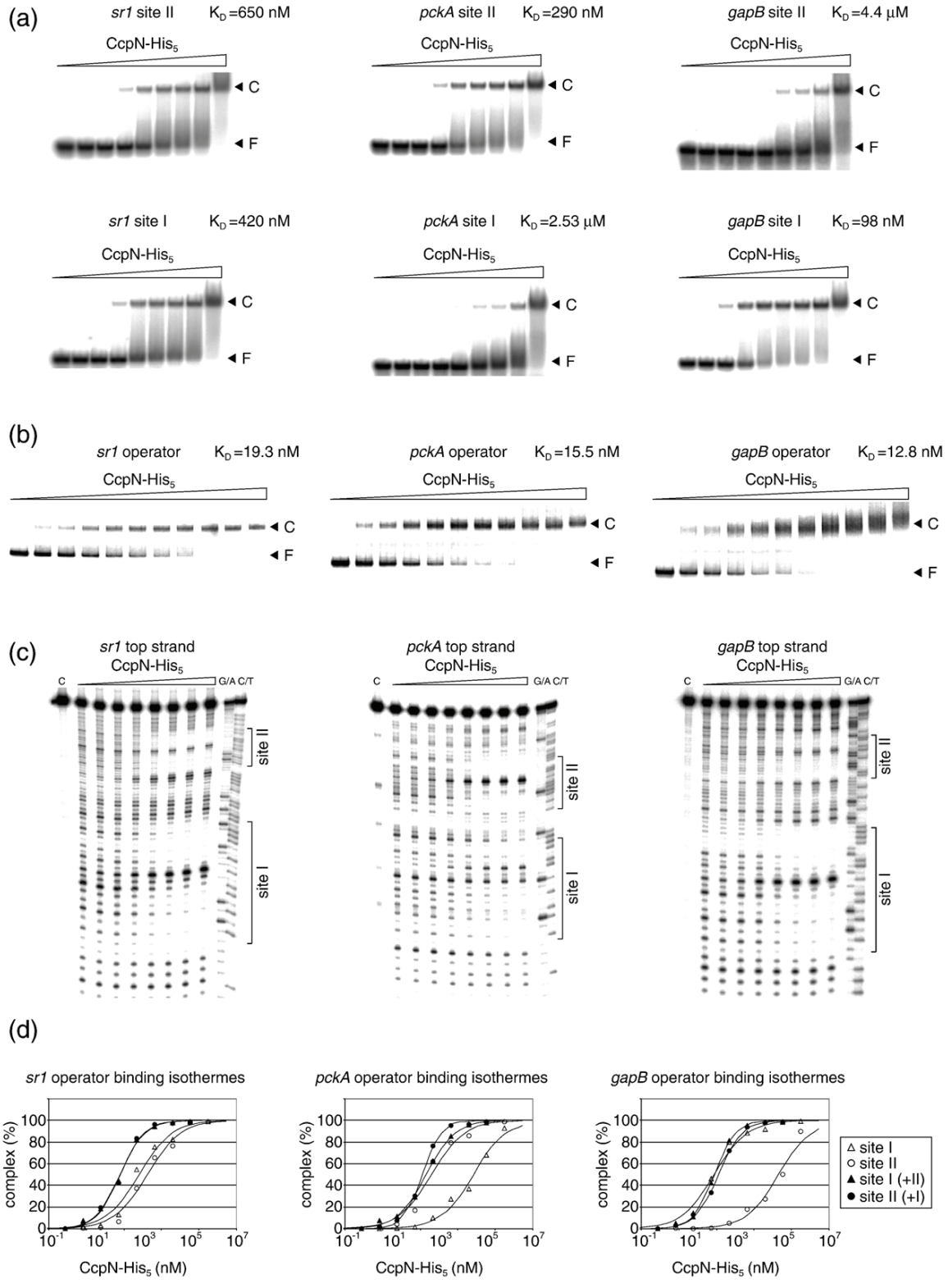

Figure 6 (*legend on opposite page*)

Table 1. Apparent dissociation constants and free energies for all CcpN binding sites

Single sites			Whole operator				
Binding site	K_D (nM)	ΔG (kJ/mol)	Binding site	K_D (nM)	ΔG (kJ/mol)	$\Delta\Delta G$ (kJ/mol)	h
sr1, site I	420 (±100)	−37.9 (±0.7)	*sr1*, site I	80 (±6)	−42.1 (±0.2)	−9.5	1.37
sr1, site II	650 (±130)	−36.8 (±0.5)	*sr1*, site II	81 (±16)	−42.1 (±0.5)		
pckA, site I	2530 (±310)	−33.2 (±0.3)	*pckA*, site I	145 (±32)	−40.6 (±0.6)	−9.8	1.45
pckA, site II	290 (±60)	−38.8 (±0.5)	*pckA*, site II	115 (±13)	−41.2 (±0.3)		
gapB, site I	98 (±12)	−41.6 (±0.3)	*gapB*, site I	89 (±6)	−41.9 (±0.2)	−9.7	1.49
gapB, site II	4400 (±15)	−31.8 (±0.0)	*gapB*, site II	114 (±48)	−41.2 (±1.0)		

Values were derived from three independent experiments. $\Delta\Delta G$ ($\Delta G_{complete} - \Delta G_{single}$) is the extra free energy that is gained when the two occupied sites are together on one DNA molecule, and h is the Hill coefficient.

fragment. To this end, 89 bp double-stranded oligonucleotides were incubated with increasing concentrations of CcpN-His_5 and, after equilibrium was reached, subjected to cleavage with DNase I (Figure 6(c)). In all experiments, the top strand was labelled, since the DNase I cleavage pattern of this strand was more homogeneous than that of the bottom strand. The degree of protection observed corresponded directly to the occupancy of the DNA by CcpN and allowed us to calculate the amount of complex formed. Apparent equilibrium dissociation constants were estimated by non-linear regression using the average data from three independent experiments. The calculated K_D values, the Hill coefficients and the binding energy ΔG for the CcpN–DNA interaction for all single sites are summarised in Table 1. Interestingly, the apparent dissociation constants for the complete operator sequences differed from those found for the investigated single sites and from the results of the footprinting experiments.

Cooperativity of CcpN binding

The K_D values for each side in the context of the whole operator were in all cases lower than for the corresponding single sites alone (see Table 1). This was especially true for binding site I of *pckA* and binding site II of the *gapB* operator. The gain in free energy upon CcpN binding to two separated single sites was lower than to two sites in a complete operator, i.e. the occupation of both sites in the operator is cooperative. This was verified by the finding that the K_D values obtained with DNA fragments spanning the whole operator are significantly lower than those obtained with single binding sites. Furthermore, when the values for the single site isotherms in the context of the whole operator were fit to the Hill equation (see Materials and Methods), the shape and the slope of the isotherms changed to a characteristic form for cooperative interactions. Moreover, the Hill coefficient h is in each case >1 (see Table 1), which is a reliable sign for cooperativity. In the case of *sr1*, where the two binding sites have nearly identical K_D values, the affinity of each site was increased by approximately equal amounts. By contrast, when one binding site was much stronger than the other, as in the case of *pckA* and *gapB*, the K_D value for the weaker binding site was improved dramatically (from 4.4 μM to 114 nM for *gapB* site II), but the K_D for the stronger binding site was mostly unaffected. A comparison of the binding isotherms for the single sites and the single-site isotherms for the complete operators that can be found in Figure 6(d) corroborates this conclusion.

Energetic calculations on CcpN-DNA interactions

Quantitative footprinting experiments like those described above were performed at 37 °C and 52 °C. The free energy ΔG was calculated on the basis of three independent experiments. Equation (1) describes the correlation between free energy (G), enthalpy (H) and entropy (S) and can be rearranged to yield equation (2), because ΔH and ΔS are independent of temperature. Thereby, T_1 and T_2 are

Figure 6. Determination of the binding isotherms for the *sr1*, *pckA* and *gapB* operators. (a) EMSAs of single CcpN binding sites. The 23 bp oligonucleotides were incubated with increasing concentrations of purified CcpN-His_5 (CcpN concentration from left to right: 0; 8.1 nM; 27.3 nM; 72.9 nM; 219 nM; 656 nM; 1.97 μM; 5.90 μM; 17.7 μM). F, free DNA; C, CcpN–DNA complex. To allow for a direct comparison with (c), EMSAs for binding sites I and II are shown in the same order as the binding sites appear in the DNase I footprinting gels. (b) EMSAs of whole CcpN operators. The 400 bp oligonucleotides were incubated with increasing concentrations of purified CcpN-His_5 (CcpN concentration from left to right: 0; 5.2 nM; 7.8 nM; 11.7 nM; 17.6 nM; 26.3 nM; 39.5 nM; 59.3 nM; 88.9 nM; 133 nM; 200 nM). F, free DNA; C, CcpN–DNA complex. The determined K_D values are given in each diagram. (c) Quantitative DNase I footprinting: C, control (uncleaved DNA); G/A, Maxam-Gilbert G > A sequencing reaction; C/T, Maxam-Gilbert C + T sequencing reaction. The 89 bp oligonucleotides containing both CcpN-binding sites were incubated with increasing amounts of CcpN-His5 (CcpN concentration from left to right: 0; 8.1 nM; 27.3 nM; 72.9 nM; 219 nM; 656 nM; 1.97 μM; 5.90 μM). Protected regions aredenoted site I and site II. (d) Binding isotherms of the single CcpN-binding sites and single-site isotherms for the whole operator sequence. Single sites I and II are represented by open triangles and circles, respectively. Filled triangles (site I) and circles (site II) designate single-site isotherms of the complete operator. The trend curves shown are averaged from three independent experiments.

310.15 K and 325.15 K, respectively, and G_1 and G_2 the free energies at the corresponding temperatures:

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

$$\Delta S = \frac{\Delta G_1 - \Delta G_2}{T_2 - T_1} \quad (2)$$

Enthalpic and entropic contributions to CcpN-DNA binding were calculated using equation (2) and are summarised in Table 2. The CcpN–DNA interaction shows a small but unfavourable change in entropy that is overcome by a strong enthalpic contribution. This combination of enthalpy and entropy ensures that the CcpN–DNA interaction has nearly the same efficiency at all temperatures that are tolerated by *B. subtilis*.

Discussion

CcpN binds asymmetrically to its two consecutive binding sites in all three operators

Here, we report the high-resolution contact probing of the transcriptional repressor CcpN bound to its operator sites. CcpN, which has been identified recently as a repressor active under glycolytic conditions, is known to regulate three genes in *B. subtilis: sr1*, encoding a small untranslated RNA,[13] and genes for two gluconeogenic enzymes, *pckA* and *gapB*.[11] Using chemical interference footprinting with different chemical probes, we determined the bases contacted by CcpN in all three operators (summarised in Figure 5).

In all cases, two binding sites were identified, one of which was always contacted more strongly than the other. In the following, this site is referred to as the strong site, whereas the other is designated the weak site. Within all binding sites, core regions can be defined that resemble the consensus binding sequence TGTG(Y/A)YATAC that was previously determined for CcpN.[13] A comparison of all core regions with this consensus is presented in Figure 5(b).

In the *sr1* operator, the upstream binding site (site I, the strong site) was found to be contacted in a slightly stronger manner, but both binding sites showed extensive contacts especially to guanine and thymine residues (Figure 5) and less close contacts to adenine and cytosine residues, and to the sugar–phosphate backbone (Figures 1, 3 and 4). Moreover, both core regions conform well to the consensus. By contrast, contact distribution was found to be completely different in the other two operators. In the case of *pckA*, the majority of contacts were concentrated in the downstream binding site (site II, the strong site), where close contacts to all bases except cytosine were found (Figure 5). At site I, the weak site, only few and less close contacts were detected. Whereas the core region of the strong site again corresponded well to the consensus sequence, the core of the weak binding site deviated significantly from the consensus. Furthermore, although only one extended site appeared in the *pckA* DNase I footprint,[11] chemical interference revealed that the *pckA* operator consists of two binding sites, too.

Similar results have been found for the *gapB* promoter, except that the upstream site (site I) proved to be the strong site. As in the case of *pckA*, the most and the closest contacts were found in the strong site in the consensus-like core region, whereas site II showed only a low level of similarity to the consensus sequence. A series of *gapB* operator mutants tested by Servant *et al.*[11] can be evaluated in the light of the data published here: They found that a $T_{(-11)}$→A mutation, located in the strong site, severely inhibited CcpN binding, which can be explained by the close contact to the adenine residue on the complementary strand that we observed. Moreover, this position was shown to be invariant in the previously determined consensus sequence.[13] This holds true for the $A_{(23)}$→G mutation too, which concerns an invariant base in the weak site. However, the observed effect was not that pronounced, since the contribution of this position is not that great in this case. By contrast, the $T_{(-14)}$→G mutation showed almost no effect on the CcpN–DNA interaction, despite the close contacts that we mapped for this position. However, this site has been shown to be more variable in the consensus sequence,[13] and one could imagine that a mutation at this site is compensated by the surrounding sequence.

In all three operators, the major contacts determined with interference footprinting were contacts to guanine and thymine residues, and all focused within a core binding region. Since guanosine is methylated at N7 in the major groove, one can conclude that CcpN contacts its operator sequences primarily, but not exclusively, through contacts in the major groove, as found previously for many other proteins, e.g. RhaS from *E. coli*.[14] Like transcription factor TyrR from *E. coli*,[15] CcpN contacts its target through a large number of bases. Contacts to the sugar–phosphate backbone make only minor contributions to the CcpN–DNA interaction and, thus, do not seem to play an important role. Most probably, extended contacts to bases relieve the necessity to interact with the sugar–

Table 2. Reaction enthalpy and entropy for the CcpN–DNA interaction

Binding site	ΔG 37 °C (kJ/mol)	ΔG 52 °C (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/(mol K))
sr1, site I	−42.3	−41.8	−51.5	−0.03
sr1, site II	−42.7	−41.5	−68.4	−0.08
pckA, site I	−40.4	−39.5	−59.2	−0.06
pckA, site II	−41.7	−41.4	−48.6	−0.02
gapB, site I	−41.7	−41.3	−49.8	−0.03
gapB, site II	−41.2	−38.4	−100.1	−0.18

Quantitative footprinting was performed at 37 °C and 52 °C with DNA fragments carrying the whole operator sequence. The values were derived from three independent experiments.

phosphate backbone. Interestingly, contacts to the sugar–phosphate backbone were found mostly downstream from one of the guanine bases that provided one of the main contacts.

The occurrence of two binding sites with different contact strengths within one operator is rather peculiar, as many proteins with two binding sites bind these sites with more-or-less equal affinity.[16–18] In this regard, however, CcpN shows similarities with PurR,[19,20] whose operators have one strong and one weak binding site, too, although the differences are not as pronounced as in the case of CcpN.

CcpN binding sites are located at different positions at each operator

Previous DNase I footprinting experiments indicated that the binding site distribution is different among the three CcpN-regulated promoters.[11,13] Here, we substantiated these findings and determined the exact borders of the single binding sites using chemical interference footprinting experiments. Figure 5 shows that in all three operators, CcpN binding sites are located at different positions relative to the transcription start site.

At the *sr1* operator, site I was found to be centred upstream of the −35 box, around −48, and site II centred around −19. Bases within the −35 box were not contacted by CcpN, and only one base of the −10 box exhibited one less close contact. In contrast, in the *pckA* operator, site I overlapped the −35 box partially and site II the −10 box completely. The *gapB* operator revealed yet another positioning of the binding sites. Here, binding site I covered the −10 box as does site II in the case of *pckA*, and site II was located downstream from the transcription start site with its centre at position +19.

Diverse distribution of operator sites is not an uncommon feature. Beside transcription factors that show conserved binding site positioning, like CytR from *E. coli*,[21] numerous transcription factors bind to operators that are located at varying positions with regard to the promoter, as does CcpN. One example is CcpA, the major factor for carbon catabolite repression in *B. subtilis*, whose binding sites, termed *cre* elements, can be positioned differently relative to the transcription start site: Depending on their regulated gene, they are found at e.g. −33, −3 or +37.[22,23] Interestingly, all these *cre* elements mediate transcriptional repression, although their respective repression mechanism has not been elucidated.

Based on the distribution of the CcpN binding sites at the three different promoters, it is tempting to speculate about different repression mechanisms.[24] In the case of *sr1*, neither the −35 nor the −10 box are covered or contacted by CcpN. This might allow RNA polymerase to bind simultaneously with CcpN to the *sr1* promoter, which would exclude repression by steric hindrance and could result in inhibition of open complex formation, e.g. as found for the MerR repressor of *E. coli*.[25] Another conceivable mechanism is inhibition of promoter clearance, as shown for protein P4 of phage ϕ29 at the viral A2c promoter.[26] In contrast, at the *pckA* and *gapB* promoters, inhibition of transcription might occur by steric hindrance of RNAP binding, since at least one binding site of these promoters completely covers the −10 box, as it is the case for the Fur protein from *E. coli* as well as many other transcriptional repressors.[27] Future experiments will focus on the elucidation of the repression mechanism of CcpN at all three operators, for which the ligand that modulates CcpN activity[13] still needs to be identified.

CcpN binds cooperatively to its two binding sites

Our interference footprinting experiments indicated that CcpN contacts its respective binding sites with different strengths, especially at the *pckA* and *gapB* promoters (Figure 6). These results were confirmed by EMSAs using oligonucleotides carrying the single binding sites. The K_D values determined varied greatly from as low as 98 nM for the strong site of *gapB* to 4.4 μM for the associated weak site. The same was true for the strong and the weak site of the *pckA* operator, whereas the K_D values for the binding sites of the *sr1* operator did not differ much with 420 nM and 650 nM for site I and II, respectively, due to the only slight differences in contacts between these sites (Figure 6(a) and Table 1). Surprisingly, the K_D values obtained in EMSAs with DNA fragments containing the whole operators were greatly reduced, up to 340-fold for the weak site of *gapB*, compared to those for the single binding sites and correlated well to what was found by Servant *et al.*[11] Obviously, two binding sites on one DNA strand dramatically increase the binding efficiency of CcpN.

The determination of the K_D values for the single site in the context of the complete operators confirmed these results. Here, in all three operators, both sites were occupied with almost the same efficiency and showed only slight variations in K_D values between the strong and the weak sites. In addition, all K_D values were, partly significantly, decreased. In the *sr1* operator, both binding sites showed an almost equal increase in binding affinity, whereas at the *pckA* and *gapB* operators, only the weak binding sites exhibited a significantly lower K_D in the complete operator. Thereby, the affinity of the strong binding sites was mainly unchanged or increased only slightly. This increase in binding affinity leads to an increase in energy gain upon CcpN binding: Binding to two sites that are in close vicinity on one DNA strand is energetically more favourable than binding to two separate strands. Furthermore, a change in the shape and slope of the binding curves, resulting from a Hill coefficient $h > 1$, which indicates cooperativity, was observed. All this leads to the conclusion that CcpN apparently binds to its operators in a cooperative way, but this cooperativity is different for the three promoters. While the *sr1* operator shows two more-or-less equal

binding sites, the *pckA* and *gapB* operators are composed of one main and one auxiliary site, and binding to the auxiliary site was found to be greatly improved in the presence of the main site. Strong and weak binding sites were observed also for the DeoR repressor–operator system in *B. subtilis*.[28] The DeoR operator consists of one full and one half binding site but, unlike CcpN, DeoR does not bind single sites.

Cooperative binding suggests an interaction between the CcpN molecules bound to the stronger and weaker sites. Conspicuously, the spacer region between the two binding sites differs between the three operators (see Figures 5 and 7). Whereas in the *sr1* and *gapB* operator it comprises three helical turns, in the *pckA* operator, only two helical turns separate the two binding sites. This indicates that, at least in the case of *sr1* and *gapB*, CcpN most likely bends its operator DNA to enable a contact between the two binding entities.

CcpN binding is driven exclusively by a strong binding enthalpy

The determination of binding constants and free binding energy showed that CcpN binding to its operator is unfavourable in terms of entropy change, i.e. entropy decreases upon CcpN–DNA interaction. This effect is overcome by a strong favourable enthalpic contribution, likely due to the numerous and close contacts made to the bases in the operator sequence. This has a clear practical consequence for *B. subtilis*: Since this species tolerates temperatures from as low as 12 °C to as high as 52 °C, a strong binding enthalpy, which is temperature-independent, and a low binding entropy change, whose contribution to total binding energy depends on the temperature, ensures that CcpN retains its binding affinity and K_D value for its operators over a large temperature scale.

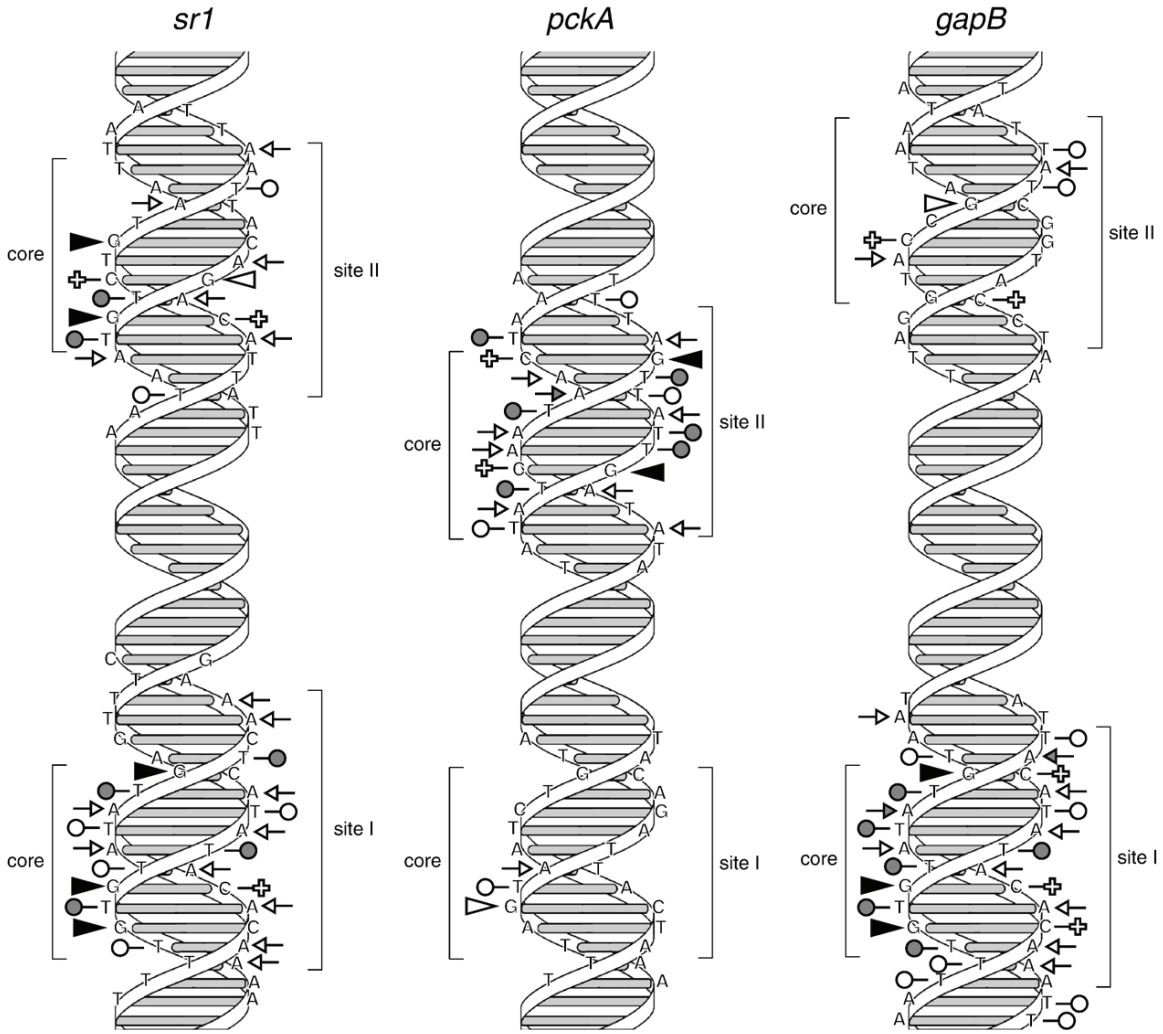

Figure 7. Ribbon model of all CcpN operators. Symbols used to indicate contacts between CcpN and the operator are the same as in Figure 5. The binding sites and the core binding regions are indicated by brackets.

Materials and Methods

Preparation of labelled CcpN targets

Oligonucleotides were purified by treatment with piperidine for 30 min at 90 °C to avoid contamination with depurinated DNA resulting from the removal of the protective groups during synthesis. Subsequently, purified oligonucleotides were 5′ end-labelled with [γ-^{32}P]ATP using bacteriophage T4 polynucleotide kinase (NEB) and purified from denaturing 15% (w/v) polyacrylamide gels.[29] Pairwise combinations of labelled and unlabelled oligonucleotides were annealed by incubation at 65 °C for 5 min and subsequent slow cooling to 37 °C. The top and bottom strand of all oligonucleotides carry two G or C residues, respectively, at each end to facilitate correct annealing and to promote additional stability. Labelled double-stranded DNA fragments for the EMSAs with the whole operator sequences were obtained by PCR using the appropriate primer pairs (all oligonucleotides used in this study are summarised in Table 3). The PCR products were purified from an ethidium bromide-stained native 6% polyacrylamide gel and 5′ end-labelled as described above. The DNA was then separated from unincorporated [γ-^{32}P]ATP by passage through a Sephadex column.

Over-expression and purification of CcpN

A *ccpN* over-expression strain was constructed by cloning a NcoI/BglII digested PCR fragment obtained with primers SB673 and SB674 on chromosomal DNA of *B. subtilis* DB104 into the pQE60 NcoI/BglII vector (Qiagen). The resulting vector was designated pQGDR. For cloning and subsequent purification of the C-terminally His-tagged protein, *E. coli* strain TG1(REP4) was used. The sequence was confirmed using a Sequenase kit (Amersham Bioscience).

A TG1(REP4, pQGDR) overnight culture grown in TY with 50 μg/ml of ampicillin and 25 μg/ml of kanamycin was diluted 100-fold, grown for an additional 3 h and induced with 1 mM IPTG. After 2.5 h, cells were harvested by centrifugation and sonicated for 10 min in sonication buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole). The supernatant obtained by centrifugation was purified over a Ni-agarose column (Qiagen). The column was washed twice with washing buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 20 mM imidazole) and, afterwards, CcpN was eluted with elution buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 250 mM imidazole). Purification was followed by SDS-PAGE. In this way, approximately 80% pure CcpN-His_5 was obtained and stored with 50% (v/v) glycerol at −20 °C.

EMSA and determination of apparent equilibrium dissociation constant K_D

Binding reactions were performed in a final volume of 10 μl containing 0.5× TBE, 0.05 g/l of herring sperm DNA as non-specific competitor, 1 nM end-labelled DNA fragment and 5.2 nM to 17.7 μM CcpN-His_5. All CcpN-His_5 dilutions were made in storage buffer and the same volume of diluted protein was used in each sample to ensure an equal concentration of salt. After incubation at 37 °C for 15 min, the reaction mixtures were separated on native 6% (for whole operator DNA fragments) or 8% (for 23 bp DNA fragments) polyacrylamide gels run at room temperature for 1 h at 200 V. Visualisation and quantification of the bands were performed using a Fuji-PhosphorImager and the PCBAS 2.09 quantification software (Raytest). All autoradiograms were made from dried gels. The image data generated by scanning the gel are linear proportionally to the radiation intensity of the sample. The amount of CcpN–DNA complex relative to the concentration of CcpN was fit with the non-linear regression programme Solver (included in Microsoft® Excel) to the following equation:

$$[C] = \frac{[D][P]}{K_D + [P]}$$

where [C], [D] and [P] represent total concentrations of formed complex, DNA and protein, respectively, and K_D is the apparent equilibrium dissociation constant.

Methylation interference footprinting

The 5′ end-labelled DNA fragments were modified by dimethyl sulphate as described for the G > A reaction using the Merck oligonucleotide sequencing kit. Modified DNA was subjected to CcpN binding and EMSA as described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and visualised by wet autoradiographic exposure. Bound and unbound DNA was cut out and eluted from the gel by diffusion (elution buffer: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.15 M NaCl), treated with phenol/chloroform and precipitated in ethanol. DNA samples and protein-free DNA as control were depurinated for 15 min at 90 °C, cleaved by piperidine for 30 min at 90 °C, precipitated in ethanol twice, resuspended in formamide loading dye and separated on a 15% polyacrylamide sequencing gel.

Potassium permanganate interference footprinting

The 5′ end-labelled DNA fragments were modified by $KMnO_4$ as described.[30] Modified DNA was subjected to CcpN binding and EMSA as described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and isolated as described above. DNA samples and protein-free DNA as control were cleaved by piperidine for 30 min at 90 °C, precipitated in ethanol twice, resuspended in formamide loading dye and separated on a 15% polyacrylamide sequencing gel.

Hydroxylamine interference footprinting

The 5′ end-labelled DNA fragments were modified by NH_2OH as dscribed.[30] Modified DNA was subjected to CcpN binding and EMSA as described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and isolated as described above. DNA samples and protein-free DNA as control were cleaved by piperidine for 30 min at 90 °C, precipitated in ethanol twice, resuspended in formamide loading dye and separated on a 15% polyacrylamide sequencing gel.

Ethylation interference footprinting

The 5′ end labelled DNA fragments were modified by N-ethyl-N-nitrosourea (Sigma) as described.[31] Modified DNA was subjected to CcpN binding and EMSA as

Table 3. Oligonucleotides used in this study

Designation	Sequence	Purpose
SB499	5′ GGAAAATGTGTTATACAGTTTGG	*sr1*, site I, upper strand
SB500	5′ CCAAACTGTATAACACATTTTCC	*sr1*, site I, lower strand
SB964	5′ GGTAAATGTGACATATTAATAGG	*sr1*, site II, upper strand
SB965	5′ CCTATTAATATGTCACATTTACC	*sr1*, site II, lower strand
SB962	5′ GGAAATAGTATAGACTATTTGGG	*pckA*, site I, upper strand
SB963	5′ CCCAAATAGTCTATACTATTTCC	*pckA*, site I, lower strand
SB602	5′ GGAATATATGTTATACTAATTGG	*pckA*, site II, upper strand
SB603	5′ CCAATTAGTATAACATATATTCC	*pckA*, site II, lower strand
SB598	5′ GGTTAATGTGTTATACTAATTGG	*gapB*, site I, upper strand
SB599	5′ CCAATTAGTATAACACATTAACC	*gapB*, site I, lower strand
SB960	5′ GGAAATTAGGATGGCATAATTGG	*gapB*, site II, upper strand
SB961	5′ CCAATTATGCCATCCTAATTTCC	*gapB*, site II, lower strand
SB869	5′ GGATATGATGATATGAAATAAAATGTGTTATACAGTTTGTTGTTGACATTTTAAATGTGACATATTAATATAATAACAACAAAAGAAGG	*sr1*, complete operator, upper strand
SB870	5′ CCTTCTTTTGTTGTTATTATATTAATATGTCACATTTAAAATGTCAACAACAAACTGTATAACACATTTTATTTCATATCATCATATCC	*sr1*, complete operator, lower strand
SB886	5′ GGATGCACACACAAAAAACAATAAATAGTATAGACTATTTGAAAATATATGTTATACTAATTCACAATTAGCAAAACACAAAAAACGGG	*pckA*, complete operator, upper strand
SB887	5′ CCCGTTTTTTGTGTTTTGCTAATTGTGAATTAGTATAACATATATTTTCAAATAGTCTATACTATTTATTGTTTTTTGTGTGTGCATCC	*pckA*, complete operator, lower strand
SB894	5′ GGTACTGGCGAATTTGTTTTAATGTGTTATACTAATTTTAGATAGTAACAAATTAGGATGGCATAATTGATAAGGGGTGTCCAACATGG	*gapB*, complete operator, upper strand
SB895	5′ CCATGTTGGACACCCCTTATCAATTATGCCATCCTAATTTGTTACTATCTAAAATTAGTATAACACATTAAAACAAATTCGCCAGTACC	*gapB*, complete operator, lower strand
SB673	5′ GAATTCCCATGGGAAGTACGATCGAACTAAAT	Plasmid pQGDR
SB674	5′ CTGCAGAGATCTTTATTAGTGATGGTGATGGTGTAGGATTTCATTTTCAGA	Plasmid pQGDR
SB342	5′ CCCAGGAGAAATTATTACAG	*sr1* downstream primer
SB423	5′ TCGAGGATCCAACAAGGTGAATATGATGAT	*sr1* upstream primer
SB1027	5′ GAGGGCAGTCAGTGCGGAGC	*gapB* upstream primer
SB1028	5′ CAATAAAAAATAAAAAGCATGCGGCTTTAAGCCGCATGCTTTTTTAGCCACAACCTCTTTGTCGT	*gapB* downstream primer
SB1029	5′ AGAGTATCCGCTCAATGAAA	*pckA* upstream primer
SB1030	5′ CAATAAAAAATAAAAAGCATGCGGCTTTAAGCCGCATGCTTTTTTTGTTGTCGCGCGAACAGCAC	*pckA* downstream primer

Oligonucleotides SB673 and SB674 were used to construct plasmid pQGDR. SB342, SB423 and SB1027-SB1030 were used as primers for the amplification of whole operator fragments. All other oligonucleotides were annealed pairwise to create double-stranded targets for footprinting experiments and EMSAs.

described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and isolated as described above. The DNA was cleaved with 143 mM NaOH at 90 °C for 30 min as described.[32] Protein-free DNA as a control was prepared by NaOH cleavage of an aliquot of the ethylated DNA. After precipitation in ethanol twice and resuspension in formamide loading dye, the samples were separated on a 15% polyacrylamide sequencing gel.

Densitometric quantification of the footprinting experiments

Band intensities were determined with quantification software (PCBAS 2.09, Raytest) and, afterwards, normalised by dividing them by the total band intensity of the same lane to correct for unequal loading. Data were plotted as logarithm (log) of the ratio of band intensity of bound DNA *versus* band intensity of the unbound DNA for each base position. Negative values were interpreted as interference signals.

Quantitative DNase I footprinting

DNase I footprinting was performed in a final volume of 10 μl containing 0.5× TBE, 6.25 mM $MgCl_2$, 0.05 g/l of herring sperm DNA, 1 nM end-labelled DNA fragment and 8.1 nM to 5.9 μM CcpN-His_5. After incubation at 37 °C for 30 min, the samples were treated with 1 μl of DNase I (Roche, 0.05 U/μl) for 2 min at 37 °C. Two control samples, one without protein, one without DNase I, were treated in parallel. The reaction was stopped by extraction with phenol and subsequent precipitation in ethanol. The pellets were dissolved in 3 μl of formamide loading dye, denatured for 5 min at 90 °C and separated on a denaturing 15% polyacrylamide gel along with a Maxam–Gilbert sequencing reaction obtained from the same DNA fragment. The dried gel was analysed by PhosphorImaging. DNA occupancy by CcpN was determined by measuring the band intensity at the binding sites divided by the intensity at an unoccupied part of the DNA.

To ensure that the CcpN–DNA complex is at equilibrium, footprinting experiments with different incubation times before DNase I cleavage were carried out. Steady state was reached no later than after 5 min of incubation. To show that DNase I is not able to displace CcpN from its operator, footprinting experiments with different concentration of DNase I were performed. The amount of CcpN-DNA complex relative to the CcpN concentration was fitted with the non-linear regression programme Solver (included in Microsoft® Excel) to the Hill equation:

$$[C] = \frac{[D][P]^h}{K_D^h + [P]^h}$$

where [C], [D] [P] and h represent total concentrations of formed complex, DNA, protein and the Hill coefficient, respectively, and K_D is the apparent equilibrium dissociation constant.

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4. Identification of ligands affecting the activity of the transcriptional repressor CcpN from *Bacillus subtilis*.

(Manuskript II)

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Identification of Ligands Affecting the Activity of the Transcriptional Repressor CcpN from *Bacillus subtilis*

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Carbon catabolite repression in *Bacillus subtilis* is mediated primarily by the major regulator CcpA. However, sugar-dependent repression of three genes, *sr1* encoding a small nontranslated RNA and two genes coding for gluconeogenic enzymes, *gapB* and *pckA*, is carried out by the transcriptional repressor CcpN (*c*ontrol *c*atabolite *p*rotein of gluco*n*eogenic genes). It has previously been shown that *ccpN* is constitutively expressed, which leads to a constant occupation of all operators with CcpN. Since this would not allow for specific regulation, a ligand that modulates CcpN activity is required. *In vitro* transcription assays demonstrated that CcpN is able to specifically repress transcription to a small extent at the three mentioned promoters in the absence of an activating ligand. Upon testing of several ligands, including nucleotides and glycolysis intermediates, it could be shown that ATP is able to specifically enhance the repressing activity of CcpN, and this effect was more pronounced at a slightly acidic pH. Furthermore, ADP was found to specifically counteract the repressive effect of ATP. Circular dichroism measurements demonstrated a significant alteration of CcpN structure in the presence of ATP at acidic pH and in the presence of ADP. Electrophoretic mobility shift assays revealed that neither ATP nor ADP altered the affinity of CcpN for its operators. Therefore, we hypothesise that the effect of ligand-bound CcpN on the RNA polymerase might be due to a conformational switch that alters the interaction between the two proteins. Based on these results, a working model for CcpN action is discussed.

Keywords: CcpN; transcriptional repressor; *in vitro* transcription; carbon catabolite repression; CD spectroscopy

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Introduction

Most bacteria, among them *Bacillus subtilis*, are able to use a huge variety of nutrients.[1,2] Nonetheless, glucose is the preferred carbon source for most of them.[3] This requires other catabolic pathways to be shut down in the presence of glucose to maximise their energy yield. This process of catabolite repression in *B. subtilis* is mediated mainly by the concerted action of CcpA and HPr-Ser46-P, which can interact to form a transcriptional regulator.[4] Though the majority of genes involved in carbon metabolism are regulated by the CcpA/HPr system, at least three genes, *gapB*, *pckA* and *sr1*, are downregulated in the presence of glucose by an alternative transcriptional repressor named CcpN (*c*ontrol *c*atabolite *p*rotein of gluco*n*eogenic genes), which exerts its function under glycolytic conditions.[5–7] *gapB* and *pckA* encode enzymes that are exclusively active during gluconeogenesis,[5,8] while *sr1* codes for a small untranslated RNA, which has been identified by a systematic search for small RNAs within intergenic regions of the *B. subtilis* genome.[9] The *sr1* gene was also found to be expressed during gluconeogenesis but repressed under glycolytic conditions. Its gene product, SR1, inhibits translation initiation of *ahrC* mRNA, encoding a transcriptional activator of the arginine catabolic operons, by a novel mechanism. Seven regions of complementarity between SR1 and *ahrC* mRNA have been found, designated A to G. Upon SR1/*ahrC* mRNA interaction, structural alterations

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Abbreviations used: CcpN, *c*ontrol *c*atabolite *p*rotein of gluco*n*eogenic genes; EMSA, electrophoretic mobility shift assay; RNAP, RNA polymerase; CTP, cytidine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.

are induced between the ahrC ribosome binding site and region G located 90 nt downstream from it. These structural alterations prevent the binding of the 30S ribosomal subunit.[10,11]

The *ccpN* gene forms a bicistronic operon with the *yqfL* gene, whose function is not yet fully clear. This operon is not autoregulated but constitutively expressed under both glycolytic and gluconeogenic conditions.[7] Homologues of CcpN have been found in the genomes of other bacilli, for example, *Bacillus halodurans*, *Bacillus cereus*, *Bacillus anthracis* and *Geobacillus stearothermophilus*, and in different firmicutes.[7]

Recent investigations have demonstrated that CcpN occupies two distinct binding sites at each of the three regulated promoters. The position of the operator sites with respect to the transcriptional start site varies depending on the promoter, but in each case, one of these sites is contacted more efficiently than the other one. However, it has been shown that both binding sites are bound with equal affinity when located in close vicinity, since CcpN binds its half-sites in a cooperative manner.[12]

The aim of the present work was to identify the ligands that modulate the activity of CcpN. Electrophoretic mobility shift assays (EMSAs) demonstrated that none of the investigated potential ligands altered the affinity of CcpN to its operator. Therefore, *in vitro* transcription assays with native *B. subtilis* RNA polymerase (RNAP) were used as an alternative method to investigate the influence of various substances on the repression activity of CcpN. These assays showed a specific increase in repression activity in the presence of high concentrations of ATP and at low pH, whereas high concentrations of ADP were able to counteract the effect of ATP. Furthermore, circular dichroism (CD) measurements that revealed a substantial ATP-induced alteration of CcpN secondary structure have been performed. The combination of these data sets allowed to develop a new working model on the mechanism of action of CcpN.

Results

In vitro transcription experiments were performed with *B. subtilis* crude extracts from a CcpN knockout strain (DB104 *ccpN::cat*)[9] that were filtrated through a Millipore column (molecular mass cutoff: 100,000 Da). This allows the separation of the RNAP holoenzyme from smaller proteins but retains any RNAP-associated factors. It has been confirmed previously that RNAP purified this way yields the same results as His-tagged *B. subtilis* RNAP purified according to the protocol of Fujita and Sadaie[13] and as native *B. subtilis* RNAP prepared according to the protocol of Sogo *et al.* (data not shown).[14]

A *ccpN/yqfL* double knockout strain was complemented with a plasmid carrying the *ccpN* gene under control of p_{Spac} to ensure that *yqfL* has no effect on the metabolic regulation of the *sr1* gene. Since the *ccpN* gene itself is not regulated,[7] this strain—after proper induction—behaves as a *yqfL* single knockout strain. Northern blot analyses revealed that this strain shows a response to glucose like the wild-type strain, although with a slightly reduced general *sr1* transcription level (Fig. S1). This corresponds perfectly to the findings of Servant *et al.*, who observed the same effects when investigating the influence of YqfL on *gapB* and *pckA* regulation.[7] Therefore, all effects observed below can be attributed to the action of CcpN alone.

CcpN is able to specifically repress transcription at the *sr1*, *gapB* and *pckA* promoters

Linear DNA molecules carrying the *sr1*, *gapB*, *pckA* or *RNAII* and *RNAIII* promoter, respectively, were incubated with increasing concentrations of CcpN and used as a template for an *in vitro* transcription reaction to determine whether CcpN *per se* is able to repress transcription without the addition of a ligand. *In vitro* transcriptions were performed with *B. subtilis* RNAP for the *sr1* and *gapB* promoters and—as a negative control—for promoters pII and pIII of streptococcal plasmid pIP510, controlling transcription of RNAII and RNAIII,[15] respectively. Since *B. subtilis* RNAP yielded only very faint bands in the case of *pckA*, *Escherichia coli* RNAP was used instead. All key experiments with the *sr1* promoter were performed with both polymerases to ensure that the results obtained with *E. coli* RNAP were comparable to those obtained with *B. subtilis* RNAP. Figure 1a shows the results, and Fig. 6 summarises all *in vitro* transcription experiments for better clarity. Once the CcpN concentration exceeded a certain threshold, all the promoters that are subject to regulation by CcpN *in vivo* revealed reduced transcription. By contrast, promoters pII and pIII, which are not subject to regulation by any *B. subtilis* protein, were not affected by CcpN even at very high concentrations. The observation that the *sr1*, *gapB* and *pckA* promoters are repressed by CcpN even in the absence of an added ligand corresponds very well to the observations made by Servant *et al.*, who reported a significant derepression of the *gapB* and the *pckA* gene in a *ccpN* knockout strain.[7]

LacZ fusions show that different glycolysis mutants influence repression by CcpN

Since the presence of glucose in the medium influences CcpN activity, we constructed transcriptional *sr1–lacZ* fusions to investigate whether intermediates of the glycolytic pathway affect CcpN. These constructs were integrated into the chromosome of *B. subtilis* strains that bear mutations in different glycolytic genes, thus interrupting glycolysis at certain steps. Strain QB5331 harbours a knockout of glucose-6-phosphate isomerase, and strain SU22 harbours a mutation in the glyceraldehyde-3-phosphate-dehydrogenase gene.[16] Both strains showed growth curves similar to the wild type (data not shown). β-galactosidase measurements, summarised in Table 1, showed that in the wild-type strain, *sr1* expression was repressed ≈37-

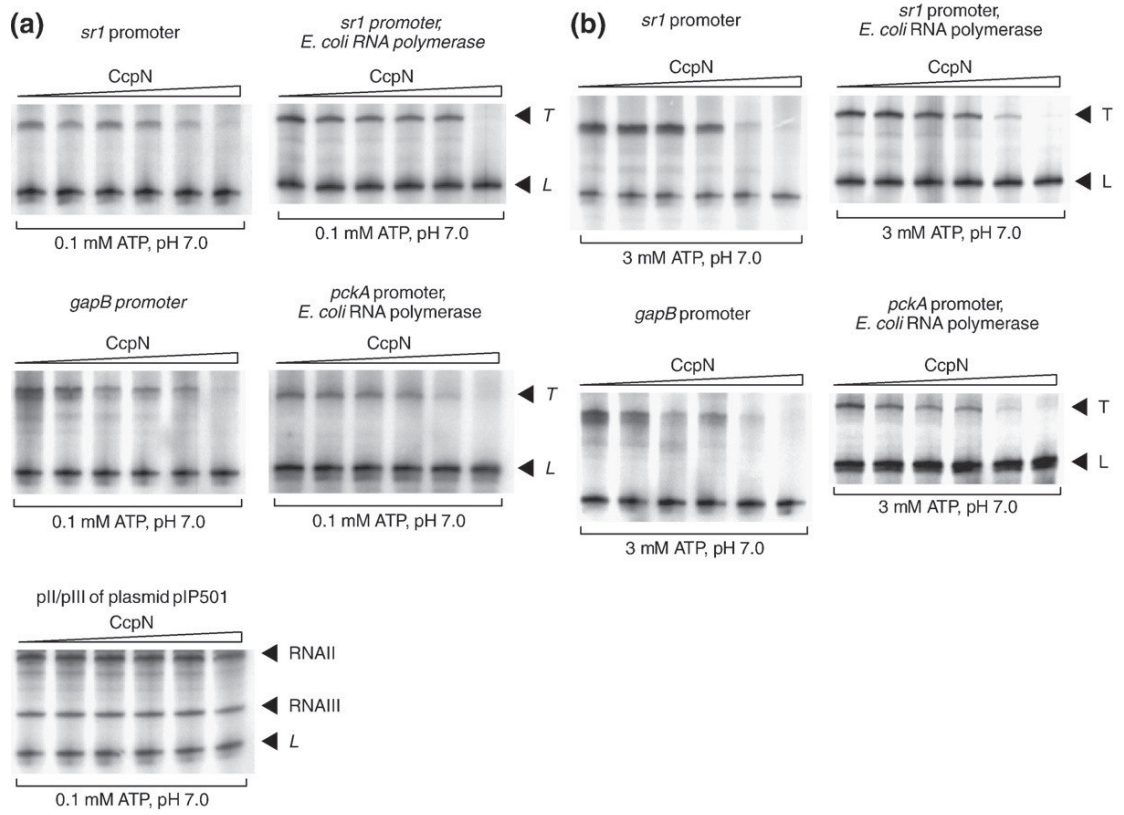

Fig. 1. Effect of CcpN and ATP on *in vitro* transcription. (a) *In vitro* transcription assay at pH 7.0 using 100 nM DNA fragment containing the promoter of *sr1*, *gapB*, *pckA* or *RNAII/III* at (a) 0.1 mM ATP or (b) 3 mM ATP. *B. subtilis* RNAP (100 nM) was used in each reaction. CcpN concentration from left to right was 0 nM, 78 nM, 156 nM, 313 nM, 625 nM and 1.25 μM, respectively. Where indicated, 50 nM *E. coli* RNAP was used. A radioactively labelled 89-nt DNA fragment served as a loading control to ensure equal amounts of the reaction being loaded onto each lane. The bands corresponding to the transcript (T) and to the loading control (L) are indicated. The autoradiograms of the gels are shown.

fold. Strain SU22 exerted a CcpN-mediated repression of factor 33, which is still significant but not as strong as the wild type. By contrast, strain QB5331 suffered from a severe lack in the ability to respond to CcpN in the presence of glucose in the medium, as it only showed a repression factor of 3.5. These results might imply that one of the glycolysis intermediates between glucose-6-phosphate and 1,3-bisphosphoglycerate is the ligand of CcpN. This hypothesis was surveyed in *in vitro* transcription assays.

Table 1. Results of β-galactosidase measurements

Strain	Mutation	−Glucose (miller units)	+Glucose (miller units)	Repression factor
DB104	None	890 (±63)	24 (±4)	37
SU22	*gapA*	523 (±5)	16 (±5)	33
QB5331	*pgi*	359 (±89)	104 (±14)	3.5

Summary of β-galactosidase measurements with wild-type *B. subtilis* and different strains with mutated glycolysis genes. Denoted mutations refer only to mutations in genes of the glycolytic pathway. Cultures were grown in SP medium to an OD_{600} of 2.0 (early stationary phase). Data are averaged from three independent experiments.

Carbon catabolism intermediates do not affect CcpN-mediated repression

In vitro transcription assays were performed in the presence of a variety of substances, including nucleotides and carbon catabolism intermediates, to test whether certain molecules, especially glycolysis intermediates, affect the repression effect of CcpN. Since some intermediates are not commercially available and others are present at very low concentrations *in vivo*,[17,18] only certain compounds were tested. A complete list of all tested substances can be found in Table 2. Since the experiments presented in Fig. 1 demonstrated that the three promoters respond to CcpN in the same manner, only the *sr1* promoter was used as a model promoter for this screening. Figure 2 shows the results of these experiments. Of all tested nucleotides, only ATP had an effect on the transcription level. It increased transcription efficiency by a factor of 5, but this effect was not related to the presence of CcpN. This increase in the presence of 3 mM ATP can be explained by the increase in stability of the open complex, since an A is the first nucleotide of all three newly synthesised transcripts. Based on these results, all other substances were tested in the

Table 2. List of all tested putative ligands in *in vitro* transcription

Substance	Relative transcription
Control	1.0×
ATP	1.0×
CTP	0.8×
AMP	0.9×
ADP	1.0×
dAMP	1.3×
dATP	0.8×
Adenosine	0.8×
GMP	0.8×
GDP	1.3×
GTP	1.2×
dGTP	0.7×
CMP	0.7×
dCMP	0.9×
UTP	1.0×
dTTP	1.3×
NADH	1.0×
NADPH	1.1×
FAD	1.2×
SAM	1.0×
Citrate	0.9×
Succinate	1.1×
Glucose	1.2×
Pyruvate	0.9×
Glyceraldehyde-3-phosphate (acidic)	0.2×
Glyceraldehyde-3-phosphate (neutral)	0.9×
Fructose-1,6-bisphosphate	1.1×
Phosphoenolpyruvate	0.8×
Glutamate	1.1×
L-Arginine	1.1×
L-Methionine	1.0×
L-Lysine	1.2×
Dihydroxyacetonephosphate	1.0×
2-Phosphoglycerate	0.9×

Summary of all substances investigated in *in vitro* transcription. Relative transcription shows the amount of transcript at 625 mM CcpN divided by the amount of transcript in the absence of CcpN. All substances were applied at 1 mM final concentration. Data are averaged from three independent experiments.
dAMP, deoxyadenosine monophosphate; dATP, deoxyadenosine triphosphate; GMP, guanosine monophosphate; GDP, guanosine diphosphate; dGTP, deoxyguanosine triphosphate; CMP, cytidine monophosphate; dCMP, deoxycytidine monophosphate; dTTP, deoxythymidine triphosphate; FAD, flavin adenine dinucleotide; SAM, *S*-adenosyl-L-methionine.

presence of high ATP concentrations to ensure reliable detection of the transcript. As can be seen in Fig. 2, the presence of glyceraldehyde-3-phosphate led to a significant decrease in transcription efficiency only in the presence of CcpN. However, closer inspection revealed that the glyceraldehyde-3-phosphate solution was acidic, causing the pH of the *in vitro* transcription buffer to drop from 7.0 to 6.5. Tests performed with neutralised glyceraldehyde-3-phosphate at low ATP concentration, at neutral and acidic pH and in the presence or absence of CcpN showed no effect at all, which attributes the specific repression effect to the acidic pH value (data not shown).

ATP specifically enhances CcpN-mediated repression at the three promoters

The search for conserved domains in the CcpN sequence revealed, beside the DNA binding domain, a pair of CBS domains.[7] These domains can be found in a variety of proteins in all three kingdoms of life and have been shown to exert different functions, such as binding of adenine nucleotides[19] and formation of an oligomerisation interface or parts of an ion transport channel.[20,21] Since binding of ATP or other adenine nucleotides would be very feasible in the case of CcpN, as it reflects the metabolic state of the cell, a series of experiments in the presence of ATP were performed. Since the results obtained at constant CcpN concentrations did not show a specific effect of ATP (Fig. 2), the CcpN concentration was varied. As can be seen in Fig. 1b, the presence of 3 mM ATP decreased the minimal inhibitory concentration of CcpN by approximately a factor of 2 at all three promoters. Regarding the efficient expression of these three genes *in vivo*, the effect was considerably smaller than expected.[7,9] Therefore, it seemed that another ligand is required for efficient repression.

Acidic pH value is the second requirement for CcpN-dependent repression

As shown in Fig. 2, low pH value in the presence of high ATP concentration led to a strong and specific repression of transcription by CcpN. To examine whether low pH value alone would be sufficient to induce CcpN-dependent repression, we performed *in vitro* transcription experiments at constant CcpN concentration in the presence of 0.1 mM ATP while pH was decreased from pH 7.2 to 6.5 (Fig. 3a). Alternatively, the effect of increasing CcpN concentration at pH 6.5 and 0.1 mM ATP was investigated (data not shown). Neither of these combinations showed any specific repression at all, implying that low pH is necessary but alone not sufficient for CcpN activity. A second set of experiments, using the same combinations of pH value and CcpN concentration, but performed at 3 mM ATP, showed a strong specific repression effect (Figs. 3b and 4a–c). This indicates that a

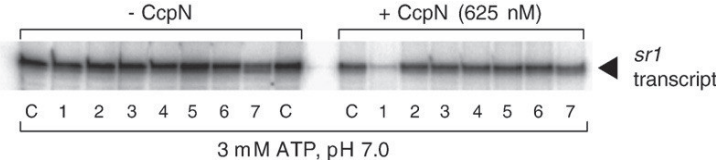

Fig. 2. Investigation of putative effectors. *In vitro* transcription assay at pH 7.0 using 100 nM DNA fragment containing the *sr1* promoter. *B. subtilis* RNAP (100 nM) was used in each reaction. All investigated substances were applied at 1 mM final concentration. C, control; 1, glyceraldehyde-3-phosphate (free acid); 2, phosphoenolpyruvate; 3, dATP; 4, pyruvate; 5, citrate; 6, fructose-1,6-bisphosphate; 7, GDP. Table 2 shows a summary of all tested substances. The autoradiogram of the gel is shown.

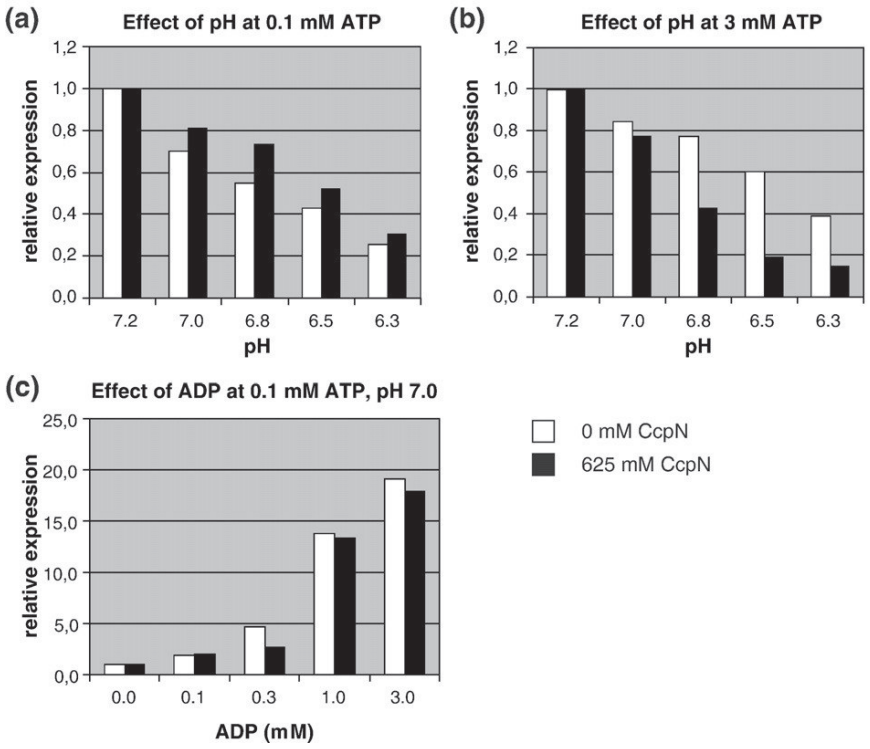

Fig. 3. Effect of ATP, low pH and ADP on *in vitro* transcription using 100 nM DNA fragment and 100 nM *B. subtilis* RNAP in each reaction. The pH was adjusted with HCl. (a) Column diagrams of *in vitro* transcription assays at different pH values using the *sr1* promoter in the presence of 0.1 mM ATP without or with 625 nM CcpN, as indicated. The relative transcript levels, normalised at pH 7.2, in the presence and absence of CcpN are shown. (b) Column diagrams of *in vitro* transcription assays at different pH values using the *sr1* promoter in the presence of 3 mM ATP without or with 625 nM CcpN, as indicated. The relative transcript levels, normalised at pH 7.2, in the presence and absence of CcpN are shown. (c) Column diagrams of *in vitro* transcription assay at different ADP concentrations using the *sr1* promoter in the presence of 0.1 mM ATP without or with 625 nM CcpN, as indicated. The relative transcript levels, normalised at 0 mM ADP, in the presence and absence of CcpN are shown.

combination of ATP and low pH is required to unfold the full repression capability of CcpN.

ADP can specifically counteract the effect of ATP to relieve repression by CcpN

Recently, the crystal structure of the regulatory domain of CcpN was solved in the group of N. Declerck who showed that CcpN is, besides binding ATP, also able to bind ADP (D. Chaix *et al.*, manuscript in preparation). Inspired by this finding, we tested whether ADP had any influence on the repression activity of CcpN in *in vitro* transcription assays. Figure 3c shows that ADP alone in addition to 0.1 mM ATP neither increased nor decreased CcpN-mediated repression. However, when equimolar concentrations of ADP were added to an *in vitro* transcription reaction performed with 3 mM ATP at pH 6.5, ADP was capable to completely counteract the repression-enhancing effect of ATP (Fig. 4d–f).

CcpN mutated in a CBS domain loses its ability to respond to ATP or ADP

To examine whether CcpN with a mutation in its nucleotide binding domain retains its ability to respond to the two nucleotides, we investigated a mutant version of CcpN in *in vitro* transcription: K127A (Fig. 5). In this mutant, a conserved amino acid within one of the two CBS domains was replaced by an alanine. The mutant was a kind gift by Stéphane Aymerich and will be published elsewhere (D. Chaix *et al.*, manuscript in preparation). Figure 6 shows that this mutant exerted the same kind of basic repression that could be observed with the wild type (Fig. 1) but lacked the ability to respond to ATP or ADP. According to Stéphane Aymerich (D. Chaix *et al.*, manuscript in preparation), this mutant is not able to repress *gapB* or *pckA* transcription *in vivo*.

Repression conditions do not change the affinity of CcpN for DNA

Since ATP and low pH value have a strong effect on CcpN activity, we wanted to analyse whether they affect the affinity of CcpN for its operator sequence. To this end, a double-stranded DNA fragment harbouring the *sr1* operator region was incubated with increasing concentrations of CcpN and subjected to an EMSA. This reaction was performed under nonrepressive conditions, in the presence of 3 mM ATP or in the presence of 3 mM ATP and a pH

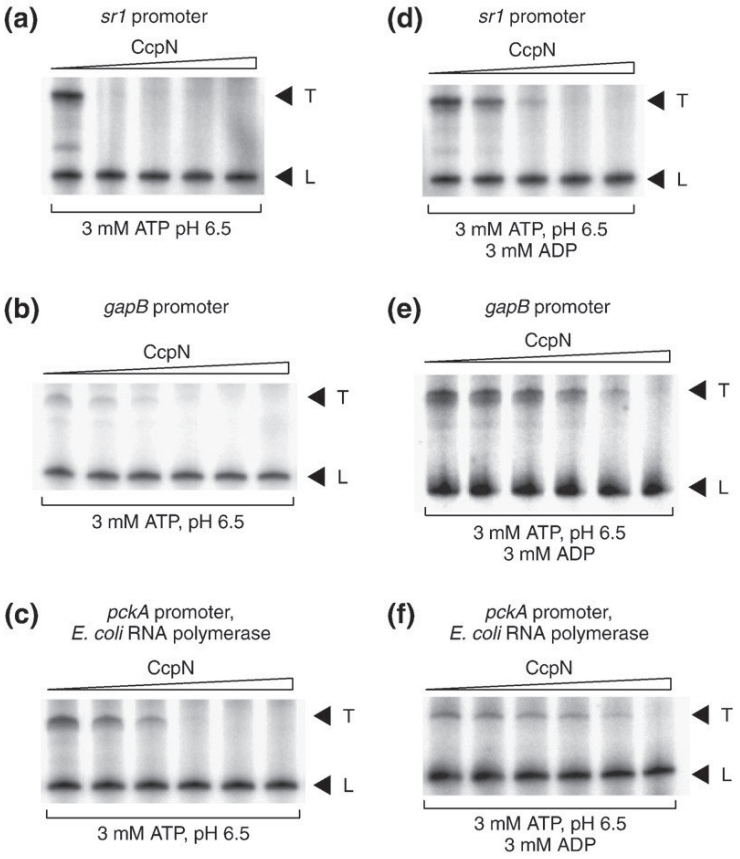

Fig. 4. Effect of ADP on *in vitro* transcription. *In vitro* transcription assay using 100 nM DNA fragment and 100 nM *B. subtilis* RNAP in each reaction. Where indicated, 50 nM *E. coli* RNAP was used. The loading control was as described in Fig. 1. The autoradiograms of the gels are shown. (a) *In vitro* transcription assay at pH 6.5 using the *sr1* promoter in the presence of 3 mM ATP with increasing concentrations of CcpN (0 nM, 313 nM, 625 nM, 1.25 μM and 2.5 μM). (b) *In vitro* transcription assay at pH 6.5 using the *gapB* promoter in the presence of 3 mM ATP with increasing concentrations of CcpN (0 nM, 78 nM, 156 nM, 313 nM, 625 nM and 1.25 μM). (c) *In vitro* transcription assay at pH 6.5 using the *pckA* promoter in the presence of 3 mM ATP with increasing concentrations of CcpN, as in (b). (d) *In vitro* transcription assay at pH 6.5 using the *sr1* promoter in the presence of 3 mM ATP and 3 mM ADP with increasing concentrations of CcpN, as in (a). (e) *In vitro* transcription assay at pH 6.5 using the *gapB* promoter in the presence of 3 mM ATP and 3 mM ADP with increasing concentrations of CcpN as in (b). (f) *In vitro* transcription assay at pH 6.5 using the *pckA* promoter in the presence of 3 mM ATP and 3 mM ADP with increasing concentrations of CcpN as in (b).

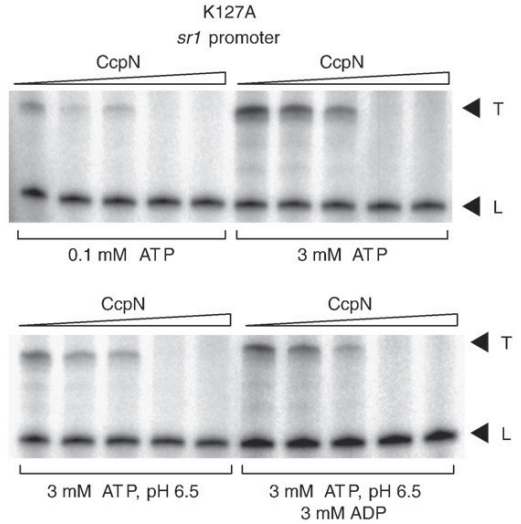

Fig. 5. Investigation of the K127A mutant in *in vitro* transcription assays. *In vitro* transcription assay at the *sr1* promoter using 100 nM DNA fragment and 100 nM *B. subtilis* RNAP in each reaction. CcpN protein with mutation K127A was added at the following concentrations: 0 nM, 313 nM, 625 nM, 1.25 μM and 2.5 μM. Reaction conditions are specified under the gel. The loading control was as described in Fig. 1. The autoradiograms of the gels are shown.

of 6.5 (Fig. 7). Both ATP and low pH, where applicable, were also present in the gel and in the running buffer to ensure that the conditions did not change during electrophoresis. However, none of the repression conditions affected the affinity of CcpN to its operators. This suggests that the specific repression of CcpN induced by ATP and an acidic pH shift is not based on an increased affinity to the promoter.

CD measurements reveal an influence of ATP and ADP on the protein structure

CD experiments were used to detect an influence of ATP or ADP on the secondary structure of CcpN. The far-UV CD spectra of the protein in the presence and absence of ATP or ADP at neutral or acidic pH are presented in Fig. 8. Without addition of a ligand, the protein displayed two negative extrema near 208 and 222 nm that indicate the presence of α-helical structures. At neutral pH, the spectrum did not alter significantly after addition of ATP. However, when ATP was added at pH 6.5, a substantial decrease in the α-helical content could be observed. This corresponds to the finding that only at acidic pH ATP was able to increase the repression efficiency of CcpN. On addition of increasing concentrations of ADP, a change in the CD spectrum of CcpN could also be observed, though not as pronounced as in

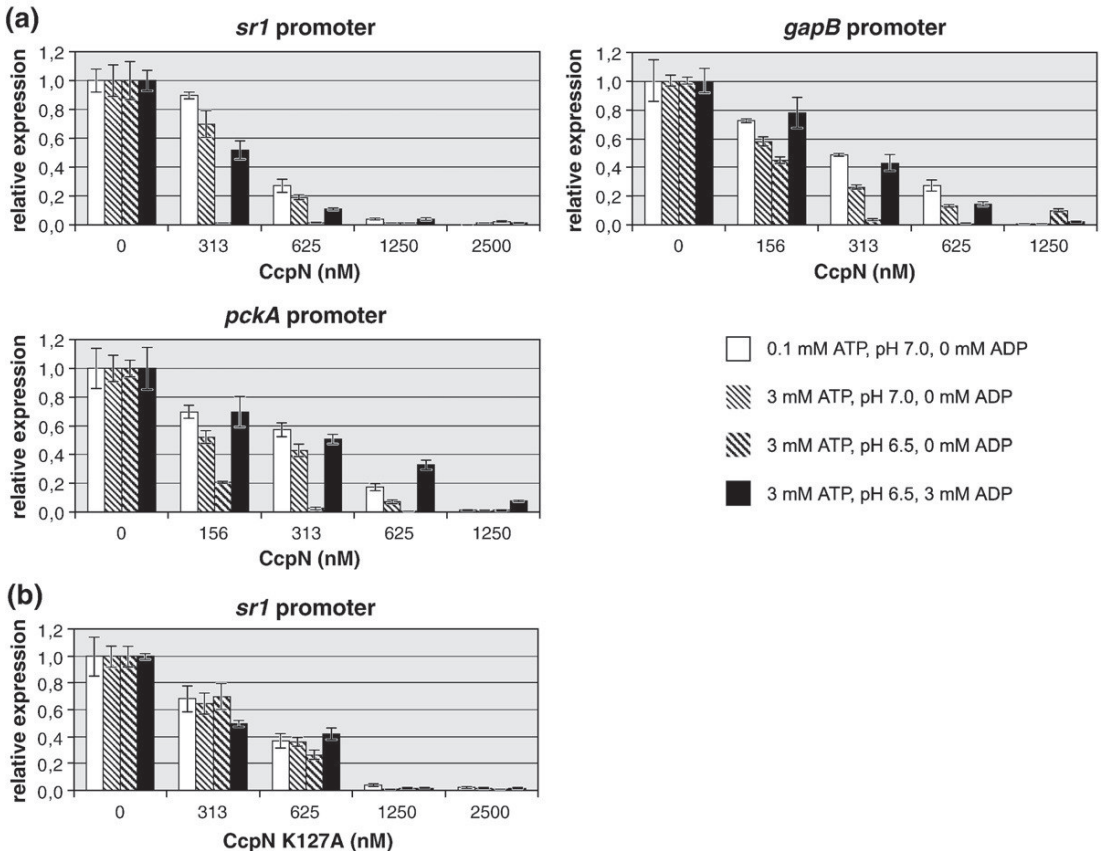

Fig. 6. Overview of *in vitro* transcriptions. (a) Summary of the effects of ATP, low pH and ADP on CcpN activity at the *sr1*, *gapB* or *pckA* promoter. Reaction conditions are indicated in the inset. Transcription levels have been normalised at 0 mM CcpN. (b) Summary of the effects of ATP, low pH and ADP on CcpN activity at the *sr1*, *gapB* or *pckA* promoter using CcpN K127A.

the case of ATP. Interestingly, the ADP effect did not seem to depend on pH, as it was almost the same at neutral and acidic conditions.

Discussion

ATP and acidic pH were identified as the two factors required for the full repression capability of CcpN

In this study, we present the identification of ligands that are necessary for CcpN to work as an efficient repressor, as well as an investigation of ligand–protein interaction. It has previously been shown that the CcpN gene is not regulated,[7] which results in a constant concentration of CcpN in the cell under both glycolytic and gluconeogenic conditions. Since CcpN-mediated repression is only required during glycolysis, a ligand is necessary to modulate its activity according to the current metabolic state of the cell.

In vitro transcription assays demonstrated that CcpN is able to exert a semi-specific repression at the three known CcpN-regulated promoters, p_{sr1}, p_{gapB} and p_{pckA}, without any ligand. By contrast, control promoters were not affected by CcpN. This finding corresponds very well to the observation of a rather

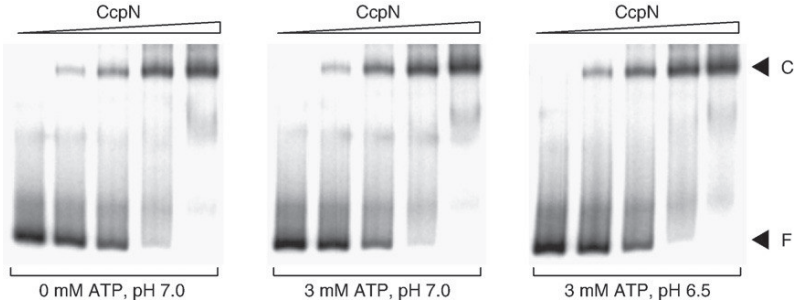

Fig. 7. EMSA with CcpN under different conditions. EMSAs of double-stranded 89-bp DNA fragments containing the *sr1* operators. The DNA was incubated with increasing concentrations of purified CcpN-His_5 (CcpN concentration from left to right: 0 nM, 156 nM, 313 nM, 625 nM and 1.25 μM). Specific reaction conditions are denoted under each gel. The autoradiograms of the gels are shown.

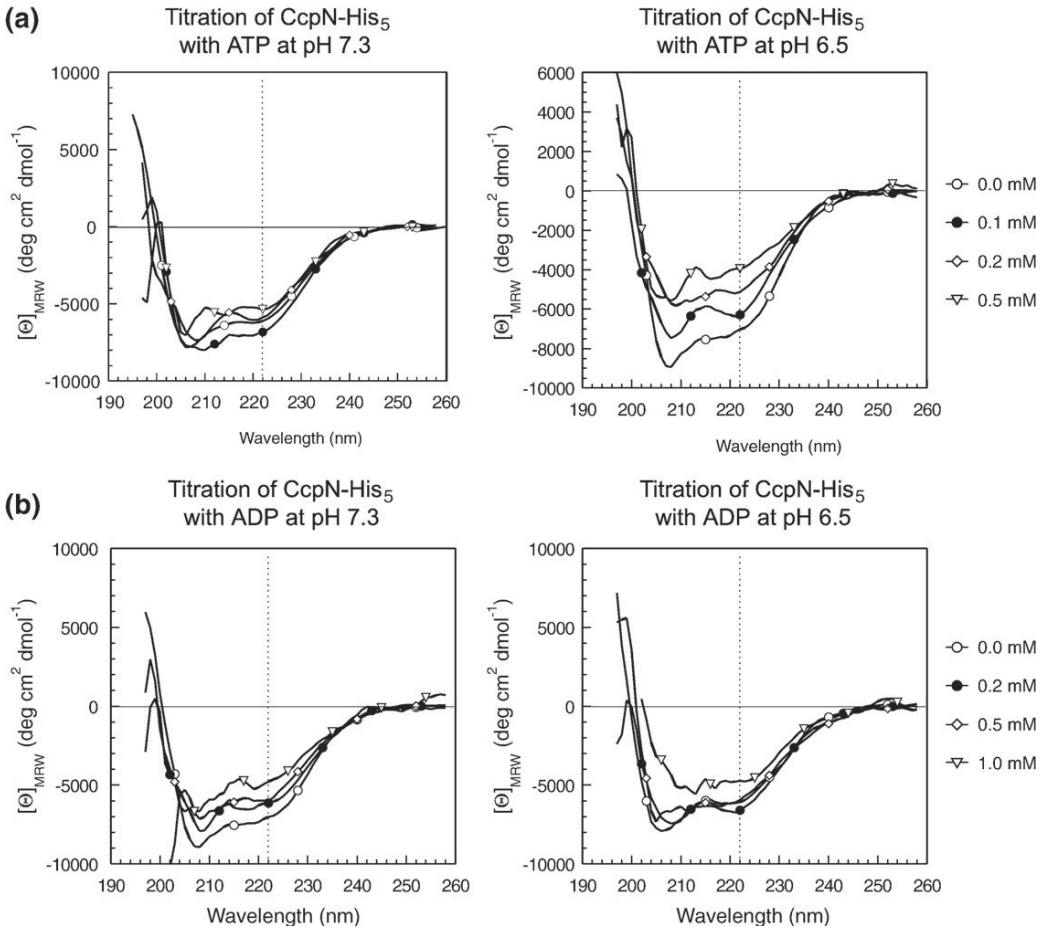

Fig. 8. Far-UV CD spectra of CcpN with ligands. CD spectrum of CcpN with increasing concentrations of ATP (a) or ADP (b) at neutral or acidic pH. The experiments were performed at 37 °C with 0.108 g/l protein.

significant derepression of p_{pckA} in a *ccpN* knockout strain.[7] Furthermore, EMSAs performed with *B. subtilis* crude protein extracts revealed a significant amount of bound DNA,[9] which might result from a high CcpN concentration in the cell. Both of these findings provide evidence that the CcpN-regulated promoters are constantly occupied by CcpN and, therefore, partly repressed in *B. subtilis*. Transcriptional regulators that are constitutively bound to their operators are not uncommon. One example is the ResD protein from *B. subtilis*, which induces the *yclJK* operon under oxygen limitation and constantly occupies a single binding site in the promoter region of this operon.[22] A similar situation can be found with the *E. coli* protein NorR, which acts as a transcriptional activator of the detoxification operon *norVW*.[23] However, none of these proteins exert a constant repression or activation upon their regulated operon, as it has been observed in the case of CcpN. It is not unusual that a transcriptional repressor knockout strain shows a slight derepression of its target gene. BzdR from *Azoarcus* sp., for example, a repressor of the anaerobic catabolism operon for benzoate, relieves its constitutive repression by a factor of ≈1.3 in the *bzdR* knockout strain.[24] Interestingly, an unspecific repression by a factor of 5.4, as exerted by CcpN, is rather peculiar, and its biological relevance remains to be elucidated.

β-Galactosidase measurements of p_{sr1}–*lacZ* fusions integrated into different strains with mutations in glycolytic genes revealed that a mutation in the *gapA* gene hardly affects CcpN-mediated repression, while a mutation in the *pgi* gene decreases repression efficiency significantly. This led to the assumption that the wanted ligand is an intermediate in this part of the pathway, namely, fructose-6-phosphate, fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate or dihydroxyacetone phosphate. However, investigation of these substances in *in vitro* transcription did not show a specific CcpN-related effect.

It has previously been shown that CBS domains, of which two are found in CcpN, are able to bind to the adenine part of nucleotides and nucleosides.[19] Surprisingly, ATP did not result in an enhanced CcpN repression in *in vitro* transcription assays with constant CcpN concentration. By contrast, when the CcpN concentration was varied, a small but reproducible effect of ATP was visible: a twofold reduction of the minimal inhibitory concentration of CcpN at all three investigated promoters. This

amount of repression was much smaller than anticipated from the *lacZ* fusions. This implies that a second factor is required for efficient repression by CcpN. Based on the results obtained with acidic GA3P, a combination of low pH and high ATP concentrations demonstrated that both of these two effectors are required to achieve full CcpN-mediated repression. The dependence on pH is not exceptional, as it is known that a specific pH is required for the correct function of many proteins, among them ion transporters and especially proteases.[25,26] It is noteworthy, however, that a pH-sensing function has only been reported for one transcription factor to date, NikR from *E. coli*, whose sensitivity to nickel is dependent on the current pH in the cell.[27] In the case of *B. subtilis* CcpN, the drop in pH in the cell might result from an accumulation of acetate as a final product of the carbon overflow mechanism,[28] which would fit well into the observed regulation performed by CcpN. If excess glucose is available, the citric acid cycle is shut down, leading to an accumulation of acetate, which is excreted afterwards, and a slight acidification of the cell.[29] This, however, does not explain the repression effect observed in the β-galactosidase measurements with strain SU22. Since none of the investigated substances showed any significant effect in *in vitro* transcription assays, the observations made with this strain might just be an artefact. It could also be possible that one of these substances exerts its effect via a hitherto unknown protein and indeed enhances CcpN-mediated repression *in vivo*.

CD measurements performed with purified CcpN-His_5 in the presence of ATP or ADP at neutral or acidic pH have strengthened the results of the *in vitro* transcription assays. Obviously, ATP binding to CcpN results in an induced fit mechanism, as significant structural changes occur when increasing concentrations of ATP are present. Such induced fit mechanisms are relatively common for ligand-binding proteins, because they are necessary for their regulatory activity. Examples include the multidrug-binding transcriptional repressor QacR from *Staphylococcus aureus*,[30] a wide range of metabolic enzymes, or the human monoamine oxidase, where structural changes have also been detected by CD spectroscopy.[31]

ADP is able to counteract the effect of ATP and HCl

We demonstrated that ADP is—at equimolar concentrations—able to specifically counteract the effect of ATP. CD measurements reinforced these findings, although the observed effect is not as strong as the effect caused by ATP (Fig. 7). Soga *et al.* have measured the intracellular concentration of metabolites and nucleotides and have shown that, while there is less ADP than ATP in exponentially growing cells, the ADP concentration exceeds the ATP concentration significantly in cells that have entered the stationary phase.[17,18] In addition to this, it is generally accepted that there is a sharp drop in intracellular ATP concentration upon glucose limitation, ultimately leading to the activation of the RsbW/RsbV system of cellular stress response.[32,33] Furthermore, it has been demonstrated that CBS domains are able to bind ADP as well as ATP,[19] which corresponds very well to our findings. It is absolutely feasible that ADP, once its concentration is high enough, replaces ATP in the binding pocket, which leads to structural rearrangements that ultimately result in a relief of CcpN repression. Such counterregulation can often be observed with enzymes that have to act differently in the presence of certain signal molecules or metabolites, such as aspartate transcarbamoylase from *E. coli* that is stimulated by ATP and inhibited by cytidine triphosphate (CTP).[34] However, transcription factors are mostly not counterregulated but have just one ligand that turns them "on" or "off," for example, BzdR and its ligand benzoyl-CoA from *Azoarcus* sp.[24] One exception, besides CcpN, is GltC from *B. subtilis*, which is activated by α-ketoglutarate and repressed by glutamate,[35] making these proteins in this respect a peculiarity. Nonetheless, there are also some differences, as GltC is also regulated by RocG *in vivo*,[36] while YqfL does not influence repression by CcpN.

CcpN with a mutation in a crucial residue can no longer exert its function

We have examined a mutation in the CcpN protein in a conserved residue within one of the CBS domains. Aymerich and Declerck (D. Chaix *et al.*, manuscript in preparation) showed that this protein is not active *in vivo* anymore. However, we observed an unspecific CcpN-mediated repression as in the wild-type case. This finding and the fact that the mutant is able to bind to its operator sequence like the wild-type protein imply that the mutation did not affect general DNA binding affinity. Furthermore, Aymerich and Declerck confirmed that this mutant has the same structure as the wild type (D. Chaix *et al.*, manuscript in preparation). Interestingly, this mutant does not respond to ATP. According to Aymerich and Declerck, mutant K127A is no longer able to bind ATP or ADP. Consequently, CcpN mutant K127A is unable to perform specific repression because of the lack of ATP binding ability.

New working model on CcpN action

It has been shown that ATP and low pH are specific effectors of CcpN in *in vitro* transcription, but they are not able to alter the binding affinity of CcpN to its operator sequence, as revealed by EMSA. Transcriptional regulators that constantly occupy their operators and share this feature with CcpN include NorR from *E. coli* or ResD, part of a two-component system from *B. subtilis*.[22,23] Such proteins usually operate through alterations in structure, induced by a ligand or another activating signal, such as phosphorylation in the case of ResD. Our CD data clearly demonstrated that at acidic pH, ATP induces significant structural rearrangements in CcpN and, therefore, strongly support this hypothesis. However, what

is the mechanism of CcpN action? Three main mechanisms can be postulated, and a general model is shown in Fig. 9: CcpN constantly occupies its operators and exerts a certain level of permanent basic repression. It is feasible that CcpN, upon ATP binding, alters its structure in a way that it occupies more space at the promoter region and replaces RNAP, resulting in a classical steric hindrance mechanism, such as that reported for the Fur protein from *E. coli*.[37] A second possibility would be that ATP-bound CcpN interacts with RNAP. This interaction could influence several phases of transcription initiation. One could imagine that an interaction between CcpN and RNAP inhibits open complex formation, as has been reported for the MerR repressor of *E. coli*.[38] A third alternative mechanism would be the inhibition of promoter clearance, as shown for protein P4 of phage ϕ29 at the viral A2c promoter.[39] It is interesting to note that, depending on the binding site, CcpN might contact different parts of the RNAP holoenzyme. CcpN bound at site I could contact the C-terminal domain of the α-subunit, while CcpN at site II might form contacts to the sigma factor. Interestingly, it is not yet clear whether two contacts to the RNAP are really necessary or even present. While it has been shown that mutations in site I completely abolish CcpN-mediated regulation,[9] this has not been proven for site II. It has been demonstrated that the binding efficiency to two binding sites is orders of magnitude larger than for single sites;[12] hence, it is conceivable that one of the sites is only an auxiliary site whose sole purpose is to increase the affinity for CcpN at this promoter. These hypotheses will be tested in future investigations.

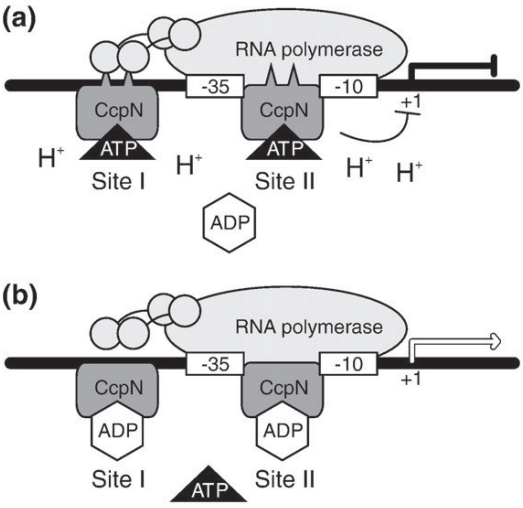

Fig. 9. Working model on the mechanism of CcpN-mediated repression. Current working model showing the effectors of CcpN. Binding sites I and II are indicated,[12] and the C- and N-terminal domains of the RNAP α-subunits are shown as spheres. (a) High glucose concentration in the medium, glycolytic conditions: ADP is only present at very low concentrations and, due to extensive glycolysis, acetate accumulates and acidifies the cell. This allows ATP to bind to CcpN, which now efficiently represses transcription. (b) Low glucose concentration in the medium, gluconeogenic conditions: ADP is present at very high concentrations and able to replace ATP in the nucleotide binding pocket. In addition to this, the citric acid cycle is no longer repressed, no acetate accumulates and the pH turns back to neutral. CcpN is no longer able to repress transcription specifically.

Materials and Methods

Enzymes and chemicals

Chemicals used were of the highest purity available. *E. coli* RNAP and all chemicals were purchased from Sigma-Aldrich™. Taq-polymerase for cloning was purchased from Roche (Germany), and Taq-polymerase for synthesis of *in vitro* transcription templates was purchased from Solis Biodyne (Estonia).

Strains, media, and growth conditions

B. subtilis strain DB104 (*ccpA*::*cat*)[9] was used for the preparation of *B. subtilis* RNAP. *B. subtilis* strains DB104, QB5331 and SU22 were used for β-galactosidase measurements. The genotypes of these strains can be found in Table 3. TY medium (16*g* Bacto tryptone, 10*g* yeast extract and 5*g* NaCl in 1 l) was used as a complex medium for the purification of RNAP. SP medium [8*g* nutrient broth, 0.25*g* $MgSO_4 \times 7\ H_2O$, 1*g* KCl, 1 ml $CaCl_2$ (0.5 M), 1 ml $MnCl_2$ (10 mM) and 2 ml ammonium iron citrate (2.2 mg/ml)] was used as a glucose-free medium for β-galactosidase measurements.

Construction of plasmids for transcriptional *lacZ* fusions

Plasmid pAC6 (Table 3) was used to insert an EcoRI–BamHI fragment obtained by PCR from chromosomal DNA of *B. subtilis* DB104 with oligodeoxyribonucleotides SB 827 and SB 831 (Table 4) to obtain a transcriptional fusion of the *sr1* promoter carrying 87 bp upstream of the −35 box and the promoterless *lacZ* gene. The resulting plasmid pACT87 was integrated into the *amyE* locus of strains DB104, QB5331 and SU22 and double crossing over was confirmed by streaking the chloramphenicol-resistant transformants on agar with 0.5% starch and subsequent overlay with iodine/potassium–iodide solution.

Construction of a plasmid for inducible *ccpN* expression

The 2.3-kb BamHI/EcoRI fragment of plasmid pPR1 containing the *repR* gene was inserted into the pOU71 BamHI/EcoRI vector, yielding plasmid pOUR. A fragment carrying the phleomycin resistance cassette flanked by EcoRI sites was generated by PCR, using primers SB 445 and SB 446 and plasmid pPR1 as template. This fragment was cloned into the pUC19 EcoRI vector, and the sequence was confirmed. The resulting vector was designated pUCP. The EcoRI fragment carrying the phleomycin resistance cassette was then obtained by restriction with EcoRI and inserted into the pOUR EcoRI vector, resulting in plasmid pOURP. Oligonucleotides SB 766 and SB 767 were annealed, yielding a polylinker sequence with 5′ BamHI and 3′ KpnI sticky ends. This fragment was

Table 3. Strains and plasmids used in this study

Strain	Relevant genotype	Reference
E. coli TG1	Wild type	Baer *et al.*[40]
B. subtilis DB104	Wild type	Kawamura and Doi[41]
B. subtilis QB5331	*trpC*2 *levR*8 *grl*-18 *amyE*::(*bglP–lacZ* phl)	Stülke *et al.*[42]
B. subtilis SU22	*gapA'*::*pMUTIN2*	Fillinger *et al.*[5]
Plasmid	**Description**	**Reference**
pAC6	pBR322-based vector for integration of transcriptional *lacZ* fusions into *amyE* locus of *B. subtilis*, Ap^R, Cm^R	Stülke *et al.*[43]
pACT87	pAC6 with p_{SR1} and 87 bp upstream of −35 box	Licht *et al.*[9]
pV6HK127A	pV6H carrying CcpN with a K127A mutation	D. Chaix *et al.*, manuscript in preparation
pOU75	pOU71-based vector for IPTG-inducible expression of *ccpN* in *trans*, $Phleo^R$	This work
pGKI	pGK13-based plasmid carrying the *lac* repressor gene, Em^R	S.B., unpublished results
pRS6	pBR322-based plasmid carrying *lacI*, Ap^R	Brantl and Behnke[44]
pPS4	pUC19-based plasmid carrying p_{Spac}, Ap^R	Brantl and Behnke[44]

inserted into the pOURP BamHI/KpnI vector, resulting in plasmid pOU72. A *lac* repressor fragment was obtained by restriction of plasmid pRS6 with BamHI and XbaI and inserted into the pOU72 BamHI/XbaI vector, resulting in plasmid pOU73. A p_{Spac} fragment was obtained by PCR, using primers SB 768 and SB 769 with plasmid pPS4 as a template. This fragment was subsequently inserted into the pUC19 XbaI/HindIII vector, and the sequence was confirmed. Thereafter, the fragment was obtained by restriction with XbaI and NcoI and inserted into the pOU73 XbaI/NcoI vector, yielding plasmid pOU74. An NcoI/NotI fragment carrying the *ccpN* gene devoid of its own promoter was generated by PCR, using primers SB 770 and SB 771 and chromosomal DNA of *B. subtilis* as a template. This fragment was cloned into the pOU74 NcoI/NotI vector, resulting in plasmid pOU75. The sequence was confirmed. Plasmid pOU75 was then used for inducible expression of *ccpN*.

Overexpression and purification of CcpN

CcpN overexpression and purification with a Ni^{2+}-NTA-agarose column were performed as published before.[9] Further purification was performed by streptomycin phosphate precipitation and dialysis against 1× TBE buffer, followed by an anion-exchange chromatography on a HiLoad Q-Sepharose 16/10 column. The protein was dissolved and dialysed against 45 mM Tris/borate buffer, pH 8.3, and applied to the anion-exchange column. Elution of CcpN-His_5 was achieved at about 200 mM NaCl by using a linear elution gradient (2 column volumes) of the same buffer containing 1 M NaCl. The purity and activity of CcpN-His_5 were verified by SDS-polyacrylamide gel electrophoresis (10%) and EMSA.

Protein concentration determination

The protein concentration of CcpN-His_5 was determined by absorption spectroscopy using a molar extinction coefficient of 5680 $l/(mol^{-1}\ cm^{-1})$ at 280 nm according to the method of Gill and von Hippel.[45]

CD spectroscopy

Far-UV CD measurements were performed on a CD spectropolarimeter J-820 (Jasco). Spectra were recorded at a scan speed of 100 nm/min, at a response time of 2 s and accumulated. The optical path length was 1 mm, and the temperature was set at 20 °C. The protein concentration was 108 μg/ml (4.4 μM) in 45 mM Tris/borate buffer, pH 8.3. The effect of the metabolites on the secondary structure of CcpN-His_5 was determined by titration of the

Table 4. Oligonucleotides used in this study

Name	Sequence	Purpose
SB 827	5′ ACG GAA TTC TGT ATG AAG AAG ATA TTG T	Construction of *lacZ* fusion
SB 831	5′ GCG GGA TCC TTT CTT TTG TTG TTA TTA	
SB 422	5′ TCG AGG ATC CAT GAA AGT TCA AGA AAA CGT	Template for *in vitro*
SB 342	5′ CCC AGG AGA AAT TAT TAC AG	transcription, *sr1*
SB 1027	5′ GAG GGC AGT CAG TGC GGA GC	Template for *in vitro*
SB 1028	5′ CAA TAA AAA ATA AAA AGC ATG CGG CTT TAA GCC GCA TGC TTT TTT AGC CAC AAC CTC TTT GTC GT	transcription, *gapB*
SB 1029	5′ AGA GTA TCC GCT CAA TGA AA	Template for *in vitro*
SB 1030	5′ CAA TAA AAA ATA AAA AGC ATG CGG CTT TAA GCC GCA TGC TTT TTT TGT TGT CGC GCG AAC AGC AC	transcription, *pckA*
SB 3	5′ GAA ATT AAT ACG ACT CAC TAT AGG AAA CAA CGA ACT GAA TAA	Template for *in vitro*
SB 4	5′ GAT ATA ATG GGT TTA CAG ATA TT	transcription, *RNAII/III*
SB 445	5′ GTG AAT TCG GCC ATT TTG CGT AAT AAG A	Construction of pOU75
SB 446	5′ GTG AAT TCG TCG ACT GAA CAG ATT AAT AAT AGA	
SB 766	5′ CCC CGC GGC CGC CCC GAG CTC CCC CCA TGG CCC TCT AGA CCC G	
SB 767	5′ GAT CCG GGT CTA GAG GGC CAT GGG GGG AGC TCG GGG CGG CCG CGG GGG TAC	
SB 768	5′ GCG TCT AGA CTA ACA GCA CAA GAG CGG AAA	
SB 769	5′ GCG AAG CTT CCA TGG GAA TTC TTA ATT GTT ATC CGC TCA CAA	
SB 770	5′ GCG CCA TGG ATG AAG TGA AAA GGT GGT GAG	
SB 771	5′ GCG GCG GCC GCT TAT TAT AGG ATT TCA TTT TCA GA	

respective chemical compounds to the protein solution. Spectra were corrected for buffer baseline containing the respective metabolite concentration.

Preparation of templates for *in vitro* transcription

Double-stranded templates for *in vitro* transcription were obtained using the corresponding primers (Table 3) in a PCR on chromosomal DNA of *B. subtilis* DB104. The PCR products were phenolised, extracted with chloroform twice and ethanol-precipitated using 15 mg/l glycogen as carrier. Pellets were washed with 80% EtOH and dissolved in aqua bidest. The preparation was analysed on an agarose gel, and subsequently, the DNA concentration was adjusted to 1 μM.

Preparation of *B. subtilis* RNAP

B. subtilis ccpN knockout strain DB104 (*ccpN::cat*)[9] was grown in TY medium to an OD_{560} of 4. Cells were then harvested by centrifugation and sonicated 10 min in sonication buffer [40 mM potassium phosphate, 10 mM ethylenediaminetetraacetic acid, 30 mM NaCl, 10 mM β-mercaptoethanol and 10 mM ethylene glycol bis(β-aminoethyl ether)*N*,*N*′-tetraacetic acid]. The supernatant obtained by centrifugation was filtrated through a 100,000-Da molecular mass cutoff Millipore column for 20 min at 6000***g*** to remove smaller proteins and exchange the sonication buffer for RNAP storage buffer (25 mM Tris/HCl, pH 8.4, 1 mM ethylenediaminetetraacetic acid, 7 mM β-mercaptoethanol and 50% glycerol). The preparation was stored at −20 °C.

In vitro transcription

In vitro transcription reactions were performed in a final volume of 10 μl in *in vitro* transcription buffer (40 mM Tris/acetate, pH 7.5, 10 mM magnesium acetate, 100 mM potassium acetate and 20% glycerol) in the presence of 0.1 mM ATP, CTP and guanosine triphosphate (GTP), 0.01 mM uridine triphosphate (UTP) and 0.011 μM [α-^{32}P]UTP. If indicated, potential ligands were added, followed by 100 nM double-stranded DNA template and 100 nM RNAP. The reaction was gently mixed and incubated for 15 min at 37 °C. One volume of formamide loading dye was added to the reaction, followed by denaturation for 5 min at 90 °C, quick cooling on ice and analysis on a 6% denaturing polyacrylamide gel. Electrophoresis was performed at 300 V/25 mA for 50 min. Gels were dried and subjected to PhosphoImaging (Fujix BAS 1000). PC BAS 2.0e software was used for quantification of the bands.

EMSAs

Binding reactions were performed in a final volume of 10 μl in *in vitro* transcription buffer (see above), 0.05 g/l herring sperm DNA as nonspecific competitor, 1 nM end-labelled DNA fragment and 156 nM to 1.25 μM CcpN-His_5. All CcpN-His_5 dilutions were made in storage buffer, and the same volume of diluted protein was used in each sample to ensure an equal salt concentration. After incubation at 37 °C for 15 min, the reaction mixtures were separated on 8% native polyacrylamide gels run at room temperature for 1 h at 230 V. Gels were dried and subjected to PhosphoImaging (Fujix BAS 1000).

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Supplementary Data

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5. The transcriptional repressor CcpN from *Bacillus subtilis* uses different repression mechanisms at different promoters.

(Manuskript III)

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THE TRANSCRIPTIONAL REPRESSOR CCPN FROM *BACILLUS SUBTILIS* USES DIFFERENT REPRESSION MECHANISMS AT DIFFERENT PROMOTERS

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CcpN, a transcriptional repressor from *Bacillus subtilis* that is responsible for the carbon catabolite repression of three genes, has been characterised in detail in the past 4 years. However, nothing is known about the actual repression mechanism so far. Here, we present a detailed study on how CcpN exerts its repression effect at its three known target promoters of the genes *sr1*, *pckA* and *gapB*. Using gel shift assays under non-repressive and repressive conditions, we showed that CcpN and RNA polymerase can bind simultaneously and that CcpN does not prevent RNA polymerase (RNAP) binding to the promoter. Furthermore, we investigated the effect of CcpN on open complex formation and demonstrate that CcpN also does not act at this step of transcription initiation. Investigation of abortive transcript synthesis revealed that CcpN acts differently at the three promoters: At the *sr1* and *pckA* promoter, promoter clearance is impeded by CcpN while synthesis of abortive transcripts is repressed at the *gapB* promoter. Eventually, we demonstrated with far western blots and co-elution experiments that CcpN is able to interact with the RNAP α-subunit, which completes the picture of the requirements for the repressive action of CcpN. On the basis of the presented results we propose a new working model for CcpN action.

CcpN, a transcriptional repressor from *B. subtilis*, is mediating CcpA-independent carbon catabolite repression of at least three genes: *sr1*, encoding a small RNA and *pckA* and *gapB* (1,2), encoding two gluconeogenic enzymes (3,4). Since its discovery in 2005, CcpN has been thoroughly investigated: Binding properties and binding motives were examined, revealing that CcpN possesses two asymmetric binding sites which are bound cooperatively and positioned differently at the three regulated promoters (5): At the *sr1* promoter, binding sites are located upstream of the -35 region and between the -35 and the -10 region, while binding sites cover the -35 as well as the -10 region at the *pckA* promoter. One operator at the *gapB* promoter overlaps the -10 region, the second one is located around +20. ATP and low pH have been identified as signals required for CcpN-mediated repression (6) and the detailed biophysical properties of CcpN-DNA interaction have been reported (7). In addition, it has been shown that CcpN is controlling central carbon fluxes in the metabolism of *B. subtilis* and that the growth defect of CcpN knockout mutants is caused by ATP dissipation via extensive futile cycling (8). It has been demonstrated that a CcpN knockout is able to increase the industrial production of riboflavin in *B. subtilis* by a deregulation of the *gapB* gene (9). However, nothing is known about the actual repression mechanism of CcpN yet.

Initiation of transcription is a stepwise process (10), beginning with binding of RNA polymerase to the promoter and formation of a loose closed complex, which is then rearranged into a tighter closed complex. This is followed by the melting of DNA around the transcriptional start site, called the open complex. RNAP can subsequently form the initiation complex and begin to transcribe the DNA, often producing short abortive transcripts resulting from failed attempts to leave the promoter. Eventually, RNAP escapes the promoter and forms the elongation complex. Transcriptional repressors can act at any of these steps, beginning with steric hindrance of RNAP binding, like the Fur protein of *Escherichia coli* (11) over the inhibition of open complex formation, like *B. subtilis* Spo0A at the *abrB* promoter (12) to the prevention of promoter clearance, as observed with the phage Φ29 protein p4 at the viral A2c promoter (13). Different mechanisms of transcriptional repression have already been reviewed in detail (14).

While steric hindrance of RNA polymerase binding does not involve direct repressor-RNAP contacts, repression of other steps in the transcription initiation process often does. In most of those cases, contacts between a transcriptional repressor and the C-terminal domain of the α-subunit of RNAP are described, as for the p4 protein at the A2c promoter or for the repressor Spx from *B. subtilis* (13,15). However, interactions with other subunits of RNAP have also been proposed, for example for the Rsd protein of *E. coli* or the main carbon catabolite mediator of *B. subtilis*, CcpA (16,17). A special case of repressors that interact with RNA polymerase subunits are anti-σ-factors. These proteins can sequester free σ-factor and are thus able to influence the expression of whole regulons (18,19).

In this work, we present a detailed analysis of the action of CcpN at all steps of transcription initiation and show that it prevents promoter clearance at the *sr1* and *pckA* promoter, while displaying a rare effect at the *gapB* promoter: It allows the formation of the open complex, but prevents the synthesis of abortive transcripts. Furthermore, we demonstrate that CcpN is able to interact with the α-subunit of RNAP and probably regulates the *sr1* and *pckA* promoters this way. Eventually, we present a new working model for CcpN-mediated transcriptional repression in regard to the specific operator positions and promoter sequences.

EXPERIMENTAL PROCEDURES

Strains and media used in this study – *B. subtilis* strain NIG2001 was used for expression of His-tagged *B. subtilis* RNA polymerase (20) and strain DB104 (21) was used for the preparation of *B. subtilis* protein crude extracts. *E. coli* strain TG1 (pREP4, pQGDR) was used for overexpression and purification of CcpN-His_5 and strain BL21 (DE3) (pETSigA) was used for overexpression and purification of His-tagged *B. subtilis* SigA (4,22). All strains were grown in TY medium (16 g Bacto tryptone, 10 g Yeast extract, 5 g NaCl in 1 l) with the respective antibiotics.

Protein purification – CcpN overexpression and purification with a Ni^{2+}-NTA-agarose column and by anion exchange chromatography was performed as published before (6). Expression and purification of His-tagged *B. subtilis* RNA polymerase and His-tagged SigA with a Ni^{2+}-NTA-agarose column was carried out according to the protocols established by Fujita and Sadaie (20,22).

Gel shift assays – Binding reactions were performed in a final volume of 10 µl in either 0.5x TBE and 10 mM $MgCl_2$ for the formation of closed complexes or in *in vitro* transcription buffer (40 mM Tris/acetate, pH 7.3, 10 mM magnesium acetate, 100 mM potassium acetate and 20 % glycerol) for the formation of open complexes, 0.05 g/l herring sperm DNA as nonspecific competitor and 1 nM endlabelled DNA fragment. Where indicated, 3 µM of CcpN-His_5, 3 µM of RNAP-His_6, 3 mM ATP, HCl to a final pH of 6.5 or 0.1 g/l of Heparin were added. After incubation at 37 °C for 15 min, the reaction mixtures were denatured and separated on 5 % native polyacrylamide gels run at room temperature for 1 h at 230 V. Gels were dried and subjected to PhosphorImaging (Fujix BAS 1000).

Open complex formation assays – Binding reactions were performed in a final volume of 10 µl in 50 mM sodium-cacodylate buffer (pH 7.3) using 1 nM of an endlabelled DNA fragment. Where indicated, 100 nM of CcpN-His_5, 100 nM of native RNAP, 3 mM ATP and/or HCl to a final pH of 6.5 were added. After 15 min incubation at 37 °C, 1 µl of DEPC (final concentration of 10 %) was added and the reaction was incubated at 37 °C for further 10 min. The reaction was stopped by the addition of 50 µl of stop solution (1.5 M NaAc, 0.1 g/l tRNA) and precipitated with ethanol, followed by dissolving of the pellet in 10 % piperidine and cleavage at the modified sites for 30 min at 90 °C. Subsequently, the cleavage reaction was precipitated with ethanol again and the pellet dissolved in formamide loading dye to a final activity of 2000 cpm/µl. Afterwards, 3 µl were denatured and separated on a 6 % denaturing polyacrylamide gel. Gels were dried and subjected to PhosphorImaging (Fujix BAS 1000).

In vitro *transcription* – *In vitro* transcription reactions at the *pckA* and *gapB* promoters were performed in a final volume of 20 µl in *in vitro* transcription buffer in the presence of 3 mM ATP, 0.1 mM CTP and GTP, 0.01 mM UTP and 0.011 µM [α-^{32}P]UTP. For the *sr1* promoter, 0.1 mM UTP and 0.011 µM [α-^{32}P]ATP were used to allow detection of abortive transcripts. Where indicated, HCl to a final pH of 6.5 and CcpN-His_5 were added, followed by 100 nM double-stranded DNA template and 100 nM RNAP-

His_6. The reaction was gently mixed and incubated for 30 min at 37 °C. Half of the reaction was ethanol-precipitated with potassium acetate to keep unincorporated [α-^{32}P]NTPs in solution and then dissolved in 10 µl distilled water. One volume of formamide loading dye was added to each half of the reaction, followed by denaturation for 5 min at 90 °C, quick cooling on ice and analysis on a 6 % denaturing polyacrylamide gel to detect full-length transcripts or on a 23 % denaturing polyacrylamide gel to detect abortive transcripts. Electrophoresis was performed at 300 V/25 mA for 50 min. Gels were dried and subjected to PhosphorImaging (Fujix BAS 1000).

Western and far western blotting – For Western blotting, samples were separated on a 15.5 % SDS-polyacrylamide gel and subsequently blotted using PVDF membrane (Carl Roth, Germany). A polyclonal antiserum from rabbit against CcpN-His_5 as primary antibody and horseradish-peroxidase-coupled anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc., USA) was used, both with a dilution of 1:2000. Blots were developed by diaminobenzidine reaction, digitised with a ScanPrisa 640U (Acer) scanner and analysed with TINA-PC BAS 2.08e software. For far western blotting, two identical sets of protein samples were separated by SDS-PAGE and subsequently blotted. SDS was then removed from the blot membranes by washing with PBST. After blocking, the part of the membranes containing the first set of samples was incubated with blocking buffer again for 1 h at RT, while the membrane containing the other set was incubated with 200 nM CcpN-His_5 in blocking buffer. Both membranes were then washed with PBST and incubated with primary and secondary antibody as described in the western blotting procedure.

Co-elution – B. subtilis DB104 was grown to an OD_{560} of 4 in 150 ml TY medium, cells were harvested, resuspended in 15 ml PBS and sonicated 3 times for 10 min. 180 µl PMSF (17 g/l in isopropanol) were added prior to sonication. After centrifugation at 4 °C, the supernatant was obtained and incubated with 300 nM of purified RpoA-His_6 , SigA-His_6 or without any protein for 1 h at RT. The samples were then purified using a Ni^{2+}-NTA-agarose column and the eluates analysed on a 15.5 % PAA-SDS-gel.

RESULTS

CcpN does not inhibit formation of the closed complex.

Transcriptional repressors can act during a variety of different steps in transcription initiation. To investigate whether CcpN exerts its repression effect by preventing RNA polymerase binding to the promoter, we performed gel shift assays using 89 bp end-labelled double-stranded DNA fragments carrying the *sr1*, *pckA*, *gapB* or *RNAIII* (as a negative control that is unable to bind CcpN) promoters (Figure 1). Purified CcpN-His_5 and purified His-tagged *B. subtilis* RNA polymerase alone and together were incubated with the labelled DNA fragment and complex formation was analysed on native polyacrylamide gels. The presence of CcpN or RNA polymerase alone resulted in a single band corresponding to the respective protein-DNA-complex at all three promoters. When both proteins were present, an additional band was visible at all three promoters, emerging from a complex of DNA, CcpN and RNA polymerase. As expected, the control promoter of *RNAIII* showed only a single band corresponding to an RNAP-DNA complex, but no CcpN-DNA-complex. All experiments were performed under non-repressive (0 mM ATP, pH 7.3) and under repressive conditions (3 mM ATP, pH 6.5) to assay if CcpN is able to prevent RNA polymerase binding to the promoter sequence. For analysis under repressive conditions, both ATP and low pH were also present in the gel and in the running buffer to ensure that the conditions did not change during electrophoresis. At all three promoters, the intensity of the band representing the CcpN-RNAP-DNA complex did not change in intensity when comparing non-repressive with repressive conditions, indicating that CcpN is not able to prevent the formation of the closed complex.

CcpN does not inhibit open complex formation.

The next step in transcription initiation is the formation of the open complex, involving melting of the DNA at the promoter region. In order to detect formation of an open complex, a double stranded DNA fragment was probed for the presence of single-stranded regions under different conditions using DEPC (Figure 2), which is known to react preferentially with single-stranded regions in B-form DNA (23). Usually $KMnO_4$ is used for detection of single-

stranded regions, but did not work under our buffer conditions. Therefore, DEPC was used, although it has the disadvantage of producing weaker signals at stacked adenosine residues. As shown in Figure 2, signals emerged at all three promoters upon addition of RNA polymerase that were not present in the negative control, where only DEPC was added. These signals persisted in the presence of CcpN (non-repressive conditions) as well as in the presence of CcpN, ATP and low pH (repressive conditions) at all investigated promoters. Thus, one can conclude that CcpN is not able to prevent formation of the open complex at any of the three promoters. To corroborate these findings, another assay for open complex formation using Heparin as a probe has been performed. Again, CcpN was not able to prevent the formation of open complexes at repressive or non-repressive conditions at any of the investigated promoters (Figure 2, bottom panels).

CcpN acts differently at the three promoters.

Since formation of the open complex is not impeded by CcpN at any promoter, it can either prevent the synthesis of abortive transcripts or promoter clearance. To investigate this issue, *in vitro* transcription reactions under non-repressive and repressive conditions were performed and analysed on two different denaturing polyacrylamide gels: 6 % gels were used to detect full-length transcripts while 23 % gels were used to detect abortive transcripts (Figure 3). Since there is no uridine within the first 11 bases of the *sr1* transcript, [α-^{32}P]ATP instead of UTP was used for labelling. This resulted in very faint bands for both the full-length and the abortive transcripts, because all *in vitro* transcription reactions were performed in the presence of 3 mM ATP necessary to observe the repressive effect of CcpN. To ensure that the observed abortive transcripts are produced by the analysed promoters rather than non-promoter sites on the template, templates with mutated promoters were investigated (Figure 3). Indeed, certain transcripts within the expected size of 3-11 nt are no longer produced from the mutated fragments, indicating that they emerge from the investigated promoters. At all three promoters, formation of full-length or abortive transcripts was not influenced in the presence of CcpN or low pH alone. Figure 3A shows that abortive transcripts are produced at the *sr1* promoter in all four lanes, even under repressive conditions, while synthesis of the full-length transcript is significantly repressed in the presence of CcpN, ATP and low pH. At the *pckA* promoter, most of the abortive transcripts are still produced during CcpN-mediated repression, however, the smallest two transcripts are lost. Nevertheless, Figure 3B clearly shows that abortive transcription in general is not affected by CcpN. A completely different picture can be found at the *gapB* promoter (Figure 3C). Here, bands corresponding to abortive transcripts are hardly or not at all detectable under repressive conditions. Thus, one can conclude that CcpN acts at the *sr1* and *pckA* promoters by preventing RNA polymerase from leaving the promoter and proceeding with transcription, while still allowing the production of short abortive transcripts. At the *gapB* promoter, however, CcpN impedes transcription initiation itself, resulting in the inability to produce abortive transcripts.

CcpN is able to interact with RNA polymerase

Since CcpN is able to prevent RNA polymerase from leaving the promoter, we wanted to find out whether this is due to a direct interaction. To this end, we purified the RNA polymerase α-subunit (RpoA) as well as the *B. subtilis* major σ-factor SigA. Figure 4A shows the two proteins, along with BSA and purified CcpN. While SigA is apparently pure, the alpha subunit contains some impurities, although in a much lower concentration than the protein itself. Two gels with identical protein samples have subsequently been subjected to far western blotting to analyse possible interactions between CcpN and these proteins (Figure 4B). The left panel shows the control blot that was only incubated with primary (anti-CcpN) and secondary antibody. As expected, CcpN itself produced a very strong signal, indicating that the antibodies work as intended. However, there are also two signals in the lane with the RpoA preparation: A very intensive signal corresponding to the largest impurity, indicating extensive antibody cross-reaction and a weak signal at the 27 kDa impurity. The RpoA band itself did not produce a signal, demonstrating that the anti-CcpN-antibody did not bind to it unspecifically. Furthermore, there were no antibody cross reactions with either SigA or BSA. The right panel shows the experiment itself, where the blot has been incubated with CcpN before the application of the first

antibody. Strikingly, a band emerges that corresponds exactly to the 39 kDa band comprised of RpoA, indicating that CcpN is able to specifically interact with the RNA polymerase α-subunit. Neither SigA nor BSA showed any interaction with CcpN at all.
To corroborate these findings, we investigated whether CcpN can be co-eluted with an α-subunit preparation. To this end, a crude extract of *B. subtilis* DB104 was incubated with RpoA-His_6 and subsequently purified using a Ni^{2+}-NTA-agarose column. As controls, RpoA-His_6 alone, the crude extract alone as well a crude extract preincubated with SigA-His_6 were purified in the same manner. Figure 4C shows the results of these experiments. It can be clearly seen that only in the case where the crude extract was preincubated with RpoA, a new band emerges that corresponds to native CcpN. As expected, this band runs marginally faster than the purified CcpN due to the lack of the His-Tag used for CcpN purification. Taken these results and the far western blot together, one can conclude that CcpN is able to specifically interact with the α-subunit of RNA polymerase.

DISCUSSION

Repression mechanism of CcpN

Here, we report the elucidation of the repression mechanism employed by the transcriptional repressor CcpN from *B. subtilis*. Gel shift assays demonstrated that CcpN does not prevent RNA polymerase binding and that both proteins can bind simultaneously to the promoter. Interestingly, CcpN and RNA polymerase, although able to bind simultaneously, appear to compete for binding to the used DNA fragments. Figure 1 clearly shows that the bands for all three complexes are significantly weaker when both proteins are present than the complexes where only one of the proteins is present. Since CcpN and RNA polymerase concentrations have been chosen to reflect their actual concentrations *in vivo* (unpublished observation, 24), it is conceivable that there is also a competition between these two proteins for promoter binding within the cell. This finding would also explain the observations made by Servant *et al.*, who reported a significant derepression of the *pckA* and *gapB* promoters in a *ccpN* knockout mutant, even under gluconeogenic conditions where CcpN is not active (3), a feature that was also reported for other transcriptional repressors, although not to such a huge extent (25).
Repressors that bind simultaneously with RNA polymerase, either at overlapping or at different sites, often repress transcription by preventing melting of DNA at the transcriptional start site, i.e. formation of the open complex. Such transcription factors are for example *E. coli* MerR at the *merT* promoter (26,27), which binds together with RNAP at opposite sites of the DNA helix, or the KorB protein of broad host range plasmid RK2 (28), whose binding sites do not overlap those of RNAP. CcpN features both versions of operator sites, some overlap with RNAP binding sites whereas some do not (5). However, open complex formation assays clearly ruled out the possibility that CcpN acts by preventing DNA melting at any of the promoters.
The inhibition of the synthesis of abortive transcripts, as observed by us at the *gapB* promoter, is a case rarely reported in literature. The H-NS protein at the *rrnB* P1 promoter or the FIS protein at the *gyrB* promoter are two examples for this kind of repression (29,30). For H-NS, a binding pattern similar to CcpN has been reported, where the operator overlaps the RNAP binding site. H-NS is then able to alter the DNA structure at this position, allowing the formation of open complexes, but preventing subsequent transcription. A similar mode of action is conceivable for CcpN at the *gapB* promoter. DNase I footprints have revealed the appearance of several hypersensitive sites upon CcpN binding at this promoter, which is usually a good indication for structural alterations of the DNA (3,4). At the *sr1* and *pckA* promoter, however, abortive transcripts are readily formed, but escape of RNA polymerase from the promoter is inhibited. Prevention of promoter clearance is usually mediated by one of two different ways: A repressor can bind downstream of RNAP and simply create a roadblock before a stable elongation complex can be formed. This has for example been shown for CcpA-mediated regulation of the *treP* gene in *B. subtilis*, and even as a prove of principle with an artificial construct using the Lac repressor (31,32). Regarding the operator positions at the *sr1* and *pckA* promoters, this mechanism appears to be highly unlikely, which favours the second alternative possibility: An interaction between the repressor molecule and parts of the RNA

polymerase. It is known that the polymerase can be stalled at promoters with close-to-consensus sequences, resulting from a extremely tight binding that subsequently makes promoter clearance very difficult (33). Transcriptional repressors, which usually bind their operator sequences with high affinity, can mimic the aforementioned effect by binding RNAP and keeping it in place. Examples for this mechanism include the phage Φ29 protein p4 at the phage A2c promoter (34) and the Gal repressor (35).

CcpN interacts with the RNAP-α-subunit

With respect to our finding that CcpN is able to specifically interact with the RNA polymerase α-subunit, we conclude that CcpN acts as a repressor at the *sr1* and *pckA* promoters by keeping RNAP in place through the aforementioned interaction. There are various reports about the α-subunit, and especially the C-terminal domain, being an interaction interface for transcriptional repressors, as mentioned above. However, interactions with the α-subunit have also been reported for activators, like CcpA at the *ackA* promoter (36,37) or SoxS during oxidative stress conditions (38). Considering the binding site position of CcpN at the *sr1* and *pckA* promoters, an interaction with the α-subunit appears very conceivable. It has been shown that up elements in *B. subtilis* have a slightly broader tolerance regarding location than in *E. coli* (39,40), reaching approximately from -40 to -66, which would position the α-C-terminal domain to be able to interact with CcpN at these promoters.

At the *gapB* promoter, however, an interaction with the α-subunit can be excluded, since both operator sites are to far downstream to allow any contact between the two proteins. Two possibilities are conceivable how CcpN exerts its action here: Either CcpN alters the DNA structure as mentioned above, or it interacts with an RNAP subunit other than the α-subunit or the σ-factor, since the first one cannot be contacted and no interaction has been detected with the latter. Reports of transcription factors that interact with e.g. the β-subunit are quite uncommon. One of these examples is the AsiA protein from bacteriophage T4 (41), another being the Rsd protein of *E. coli* (42), both of which have been shown to be able to interact with the core RNA polymerase. If CcpN actually interacts with parts of the RNAP other than the α-subunit needs to be experimentally determined. However, the relatively small size of CcpN, leaving not much space for extensive interaction surfaces and the fact that DNA structure is altered upon CcpN binding seem to favour the possibility of repression by DNA-structure rearrangements.

The example of CcpN shows that one single repressor can exert repression in very different ways, depending on how its operators are positioned relative to the RNA polymerase binding sites. Varying binding site distribution is quite common, found e.g. in the case of CytR from *E. coli* (43) and many more. Interestingly, cases where variations in operator positioning result in different repression mechanisms have not been frequently reported in literature. However, this is mostly because the actual repression mechanism for these proteins has not been elucidated. A well documented example where operator site positions have an impact on the repression mechanism is *cre* element positioning, allowing CcpA to exert a broad range of repression or even activation mechanisms on its targets (17).

Taking all results together, a quite clear picture of the repression mechanism of CcpN can be established where CcpN and the α-subunits are in a spatial position that allows interaction and subsequent promoter arrest at the *sr1* and *pckA* promoters, but not at the *gapB* promoter. Here, repression by modification of the DNA structure appears to be a probable alternative.

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FOOTNOTES

We are very grateful to M. Salas for the gift of native purified *B. subtilis* RNA polymerase as well as for the strain for the overproduction of His-tagged α-subunit. Furthermore, we would like to thank M. Fujita, who kindly sent us the strain for overproduction of His-tagged σ-factor. This work was supported by grant BR1552/6-3 from Deutsche Forschungsgemeinschaft (to S. B.). A. L. was financed by a scholarship from the federal state of Thuringia and by the Deutsche Forschungsgemeinschaft.

FIGURE LEGENDS

Fig. 1. EMSAs with His-tagged CcpN and RNA polymerase at the *sr1* (A), *pckA* (B), *gapB* (C) and *RNAIII* (D) promoters. The presence or absence of 3 µM CcpN-His_5 and 3 µM RNAP-His_6 is indicated above each lane. Experiments were performed under non-repressing (pH 7.0; 0 mM ATP) or repressing (pH 6.5; 3 mM ATP) conditions. Autoradiograms of the gels are shown. F: free DNA, CD: CcpN-DNA-complex, RD: RNAP-DNA-complex, CRD: CcpN-RNAP-DNA-complex.

Fig. 2. Open complex formation assay at the *sr1* (A), *pckA* (B) and *gapB* (C) promoters. Probing with DEPC is shown at the top while the corresponding Heparin-probing is shown below. For DEPC-probing, DEPC (10 %), RNAP (100 nM), CcpN-His_5 (100 nM), were added where indicated. Bands showing the presence of single stranded DNA regions and therewith open complexes are indicated by arrows. G: G>A sequencing reaction, C: C+T sequencing reaction. Positions of +1, the -10 and -35 box are indicated. Please note that the noncoding strand was used for *sr1* and *pckA*, while the coding strand was used for *gapB*. For Heparin-probing, Heparin (0.1 g/l) CcpN-His_5 (3 µM), RNAP-His_6 (3 µM) ATP (3 mM) or HCl (to a final pH of 6.5) were added where indicated. F: free DNA, CD: CcpN-DNA-complex, RD: RNAP-DNA-complex, CRD: CcpN-RNAP-DNA-complex. Autoradiograms of the gels are shown.

Fig. 3. *In vitro* transcription and detection of abortive transcripts at the *sr1* (A), *pckA* (B) and *gapB* (C) promoters. Transcription was performed in *in vitro* transcription buffer (see experimental procedures) using 100 nM DNA template and 100 nM His-tagged *B. subtilis* RNA polymerase. 300 nM CcpN-His_5 was added or pH was lowered where indicated. Half of each reaction was separated on either a 6 % denaturing polyacrylamide gel to detect the full length transcripts, indicated by an arrow or on a 23 % denaturing polyacrylamide gel to detect abortive transcripts, indicated by a bracket. Control experiments to the right of each panel show which of the abortive transcripts are produced by the investigated promoters. WT: wild-type promoter, MUT: mutated promoter, where the -10 regions have been replaced by the sequence GCCGAT (*sr1*) or GCCGCT (*pckA* and *gapB*). The estimated size of the abortive transcripts on each gel is indicated by arrows. Autoradiograms of the gels are shown.

Fig. 4. CcpN-RpoA interaction studies. The corresponding molecular weights of the marker bands are indicated beside the marker lanes. (A) 15.5 % SDS-polyacrylamide gel of different purified proteins. MW: molecular weight marker; σ: purified SigA-His_6; α: purified RpoA-His_6; BSA: bovine serum albumin; CcpN: purified CcpN-His_5. 1 µg of each protein was loaded into each lane. (B) Far western blot of the protein gel shown in (A). Equal amounts of protein were loaded into lanes 1-4 and 5-8, respectively. Proteins were renatured after blotting by washing with SDS-free PBS. Lanes 1-4 are control lanes and were just blocked, incubated with rabbit-anti-CcpN antibody and subsequently with horseradish peroxidase coupled anti-rabbit antibody. Lanes 5-8 are the sample lanes and were treated like lanes 1-5, but were incubated with 200 nM CcpN-His_5 after blocking and before incubation with anti-CcpN antibody. The blots were developed using horseradish peroxidase catalysed conversion of diaminobenzidine. PC-BAS 2.08e software was used for quantification. (C) Co-elution of RpoA-His_6 and CcpN. The lanes were loaded as follows: CE+α: RpoA-His_6 preincubated with *B. subtilis* DB104 protein crude extract (see experimental procedures) and subsequently purified using a Ni^{2+}-NTA-agarose column; α: RpoA-His_6 without preincubation with *B. subtilis* DB104 protein crude extract; CE: *B. subtilis* DB104 protein crude extract, purified; CE+σ: SigA-His_6 preincubated with *B. subtilis* DB104 protein crude extract and subsequently purified; CcpN: purified CcpN-His_5. Equal amounts of eluate were loaded into each lane.

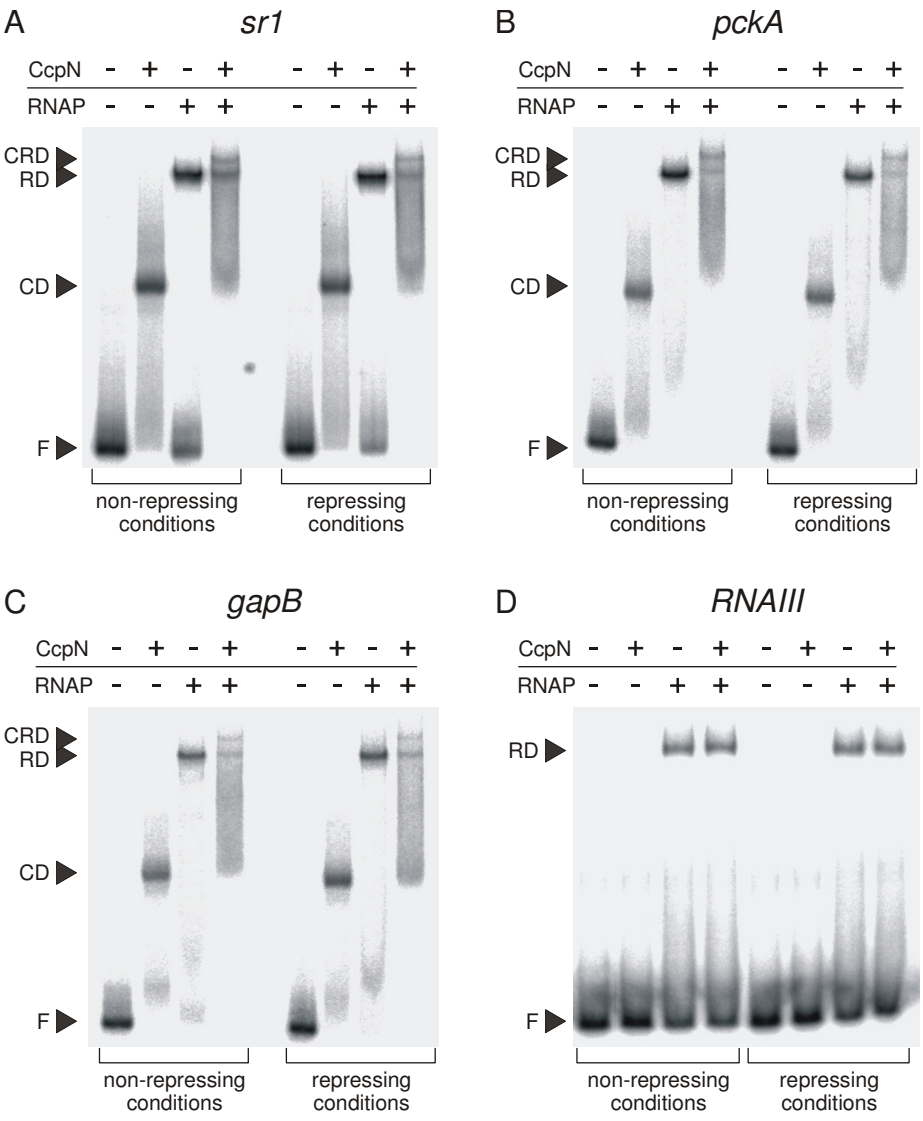

Figure 1 Licht *et al.*

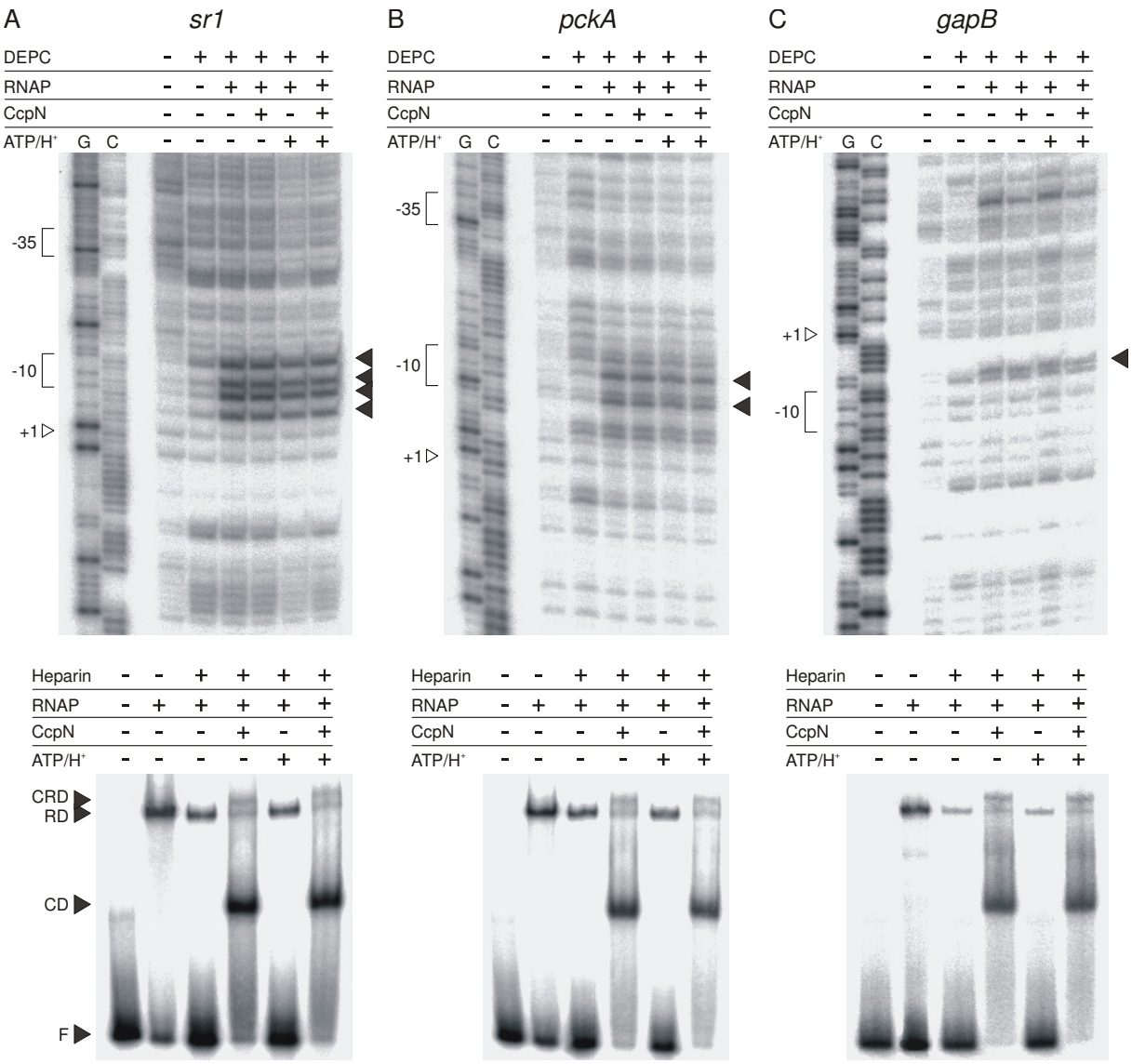

Figure 2 Licht *et al.*

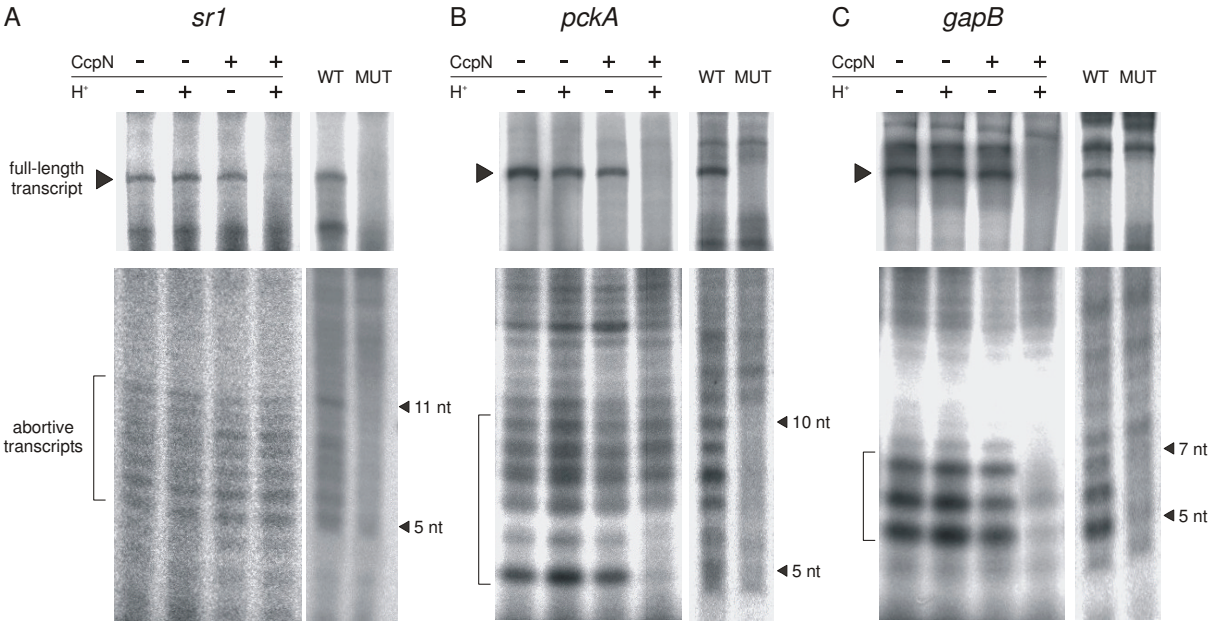

Figure 3 Licht *et al.*

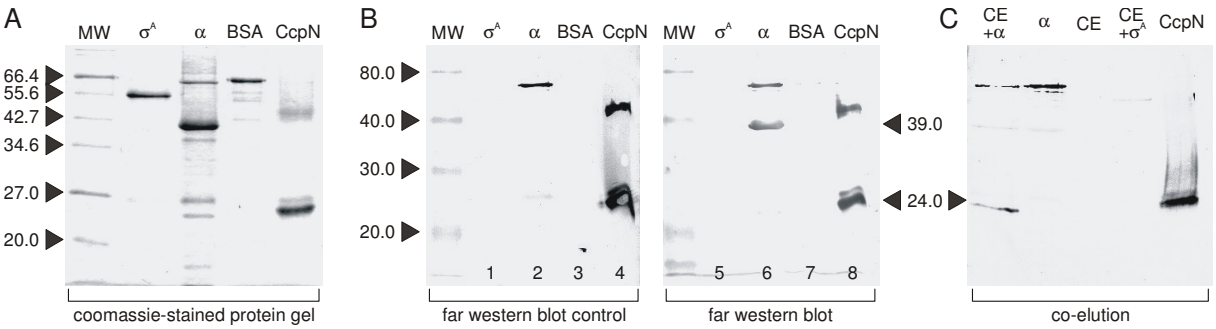

Figure 4 Licht *et al.*

6. Search for additional targets of the transcriptional regulator CcpN from *Bacillus subtilis*.

(Manuskript IV)

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SEARCH FOR ADDITIONAL TARGETS OF THE TRANSCRIPTIONAL REGULATOR CCPN FROM *BACILLUS SUBTILIS*

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Abstract

Transcriptional repressor CcpN from *B. subtilis* mediates the CcpA-independent catabolite repression of three genes, *sr1* encoding a small regulatory RNA, and two gluconeogenesis genes, *gapB* and *pckA*. The intracellular concentration of CcpN was determined to be around 4000 molecules per cell. The *B. subtilis* genome was scanned for potential new CcpN target genes, out of which three showed CcpN binding activity in their upstream region. EMSAs demonstrated that the promoter regions of two putative targets, *thyB* encoding thymidylate synthase B and *yhaM* encoding a 5'-3' exoribonuclease, bound CcpN with significant affinity. A detailed contact probing of CcpN-DNA interactions revealed an interesting new binding pattern at the *thyB* promoter, where the whole promoter appears to be contacted by CcpN. Using *lacZ*-reporter gene fusions and *in vitro* transcription assays, the *thyB* promoter was investigated for a regulatory effect of CcpN. Surprisingly, CcpN does not repress transcription at this promoter, but instead acts as an activator. Alignments of the *thyB* promoters of different Gram-positive bacteria encoding CcpN revealed CcpN consensus binding sites in a significant number of them. Our data show that a bioinformatics-based approach combined with *in vivo* and *in vitro* experiments can be used to identify new targets of transcriptional regulators.

Keywords: CcpN / transcriptional regulator / carbon catabolite repression / footprinting / alignment

Introduction

Catabolite repression is an important regulatory aspect in a variety of bacteria, among them *Bacillus subtilis* (Chambliss, 1993; Steinmetz, 1993). In *B. subtilis*, this process is mediated primarily by the combined action of CcpA and HPr-Ser46-P by forming a transcriptional regulator upon interaction (Chambliss, 1993; Stülke & Hillen, 2000). However, carbon catabolite repression of at least three known genes, *gapB*, *pckA* and *sr1*, is mediated by the transcriptional repressor CcpN (Licht *et al*., 2005; Servant *et al*., 2005). CcpN binds cooperatively to two distinct binding sites at each of these promoters and has recently been shown to require ATP and a slightly acidic pH for the exertion of its repression effect, while ADP was able to counteract the ATP-mediated repression (Licht & Brantl, 2006; Licht *et al*., 2008). The repression mechanism of CcpN has not been elucidated so far.

Bacterial transcriptional regulators can act as pure activators, e.g. MalT, the activator of maltose metabolic genes, or PhoB, an activator controlling phosphate uptake in *Escherichia coli* (Schlegel *et al*., 2002; Yamada *et al*., 1989), as pure repressors repressors like the Arg or Lac repressor from *E. coli* (Maas, 1994; Lewis, 2005) or as dual regulators acting either as activator or repressor as e.g. the global regulators CcpA and CodY from *B. subtilis* (Henkin, 1996; Sonenshein, 2005).

The number of genes regulated by a transcription factor varies widely: Some of them regulate only one single gene or operon, like the Lac repressor. These regulators are often present in rather low intracellular concentrations, e.g. 10-20 tetramers in case of the Lac repressor (Lin &Riggs, 1975; von Hippel *et al*., 1974) Others, like MalT, regulate a small set of genes or operons (Schlegel *et al.,* 2002), while others regulate a significant amount of genes as e.g. NarL that is - together with 6 other regulators - responsible for the control of 50 % of all genes in *E. coli* (Stewart, 1994).

Hitherto, CcpN could be characterised as a pure repressor controlling a small set of genes,

and although efforts have been made to identify more targets of CcpN by microarray analysis and comparative transcriptome analysis, these attempts had remained unsuccessful (Servant *et al*., 2005, Tännler *et al*., 2008).

The aim of the present work was to identify potential new targets for CcpN. One promising new target, the *thyB* gene, has been investigated in detail. We demonstrate that CcpN is able to modestly activate transcription from the *thyB* promoter. Our results show that bioinformatics in combination with experimental methods is a powerful tool to identify new targets of transcriptional regulators.

Materials and Methods

Enzymes and chemicals
Chemicals used were of the highest purity available. *E. coli* RNA polymerase and all chemicals were purchased from Sigma-Aldrich™. Taq-polymerases were purchased from Roche (Germany) and Solis Biodyne (Estonia).

Strains, media and growth conditions
B. subtilis strains DB104 (Kawamura & Doi, 1984) and DB104 (*ccpN*::*cat*) (Licht *et al*., 2005) were used. TY medium (Licht *et al*., 2005) served as complex medium. SP medium (Preis *et al.,* in press) served as glucose-free medium. Strain NIG2001 was used for expression of His-tagged *B. subtilis* RNA polymerase (Fujita & Sadaie, 1998b). *E. coli* strain DH5α was used for cloning and strain BL21 (DE3) (pETSigA) for overexpression and purification of His-tagged *B. subtilis* SigA (Fujita & Sadaie, 1998a).

Overexpression and purification of proteins
CcpN overexpression and purification were performed as published before (Licht *et al.,* 2008). Expression and purification of His-tagged *B. subtilis* RNA polymerase and His-tagged SigA were carried out as described (Fujita & Sadaie, 1998a, 1998b).

Determination of the intracellular concentration of CcpN in B. subtilis
The intracellular concentration of CcpN was determined following the procedure described for CopR (Steinmetzer *et al.,* 1998) except that the Western blot was developed with diaminobenzidine.

EMSAs and footprinting experiments
EMSAs, methylation and potassium permanganate interference footprinting were performed as described (Licht & Brantl, 2006). DNase I footprinting was performed as described (Licht *et al*., 2005).

Construction of plasmids for transcriptional lacZ *fusions and measurements of* β-galactosidase activities
Plasmid pAC6 was used to insert an *Eco*RI-*Bam*HI fragment obtained by PCR from chromosomal DNA of *B. subtilis* with oligodeoxyribonucleotides SB1069 (Table S1) and SB1070 yielding plasmid pTHY1. For plasmid pATM2, oligodeoxyribonucleotides SB1268 and SB1069 as well as SB1267 and SB1070 were used on chromosomal DNA of *B. subtilis* DB104 as template to create fragments MUT2up and MUT2down, respectively. A second PCR using these fragments and oligodeoxyribonucleotides SB1069 and SB1070 resulted in fragment MUT2, carrying the *thyB* promoter and a mutated CcpN operator site, which was inserted as an *Eco*RI-*Bam*HI fragment into plasmid pAC6. Integration of the plasmids into the *amyE* locus and measurements of β-galactosidase activities were performed as described previously (Brantl, 1994).

In vitro *transcription*
In vitro transcription reactions were performed in a final volume of 10 µl in *in vitro* transcription buffer (40 mM Tris/acetate, pH 7.5, 10 mM magnesium acetate, 100 mM potassium acetate and 20% glycerol) in the presence of 1 mM GTP, 0.1 mM ATP, 0.1 mM CTP, 0.01 mM UTP and 0.011 µM [α-^{32}P]UTP with templates generated as described (Licht *et al*., 2008). If appropriate, effectors were added, followed by the addition of 100 nM of double-stranded DNA template and 50 nM of His-tagged *B. subtilis* RNA polymerase and 50 nM SigA-His_6 and incubation for 30 min at 37 °C. Samples were treated with formamide loading dye and separated on a 6 % denaturing polyacrylamide gel at 300 V/25 mA for 50 min. Dried gels were subjected to PhosphorImaging as above.

Results and discussion

CcpN is an abundant protein in B. subtilis
To determine the intracellular concentration of CcpN, protein crude extracts from *B.*

subtilis DB104 in TY – together with purified CcpN of known concentration – were analysed by western blotting as described in Materials and Methods (Fig. 1). Since CcpN is constitutively expressed in log and stationary phase, cultures from OD_{560} = 4.0 were used. The amount of CcpN was calculated to be 4000 ± 600 molecules per cell. Taking into consideration a *B. subtilis* cell volume of 1×10^{-15} l (Abril *et al*., 1997), the intracellular concentration of CcpN is approximately 6.6 µM. Intracellular amounts of transcriptional regulators vary from few molecules, e.g. 10-20 in case of the Lac repressor from *E. coli* (Lin & Riggs, 1975), to approximately 15000 like in the case of CopR, a transcriptional repressor regulating the copy number of streptococcal plasmid pIP501 (Steinmetzer *et al.,* 1998). The pleiotropic regulators CcpA or CodY from *B. subtilis* are present in amounts resembling the one of CcpN, namely 3000 (Miwa *et al*., 1994) and ≈2500 (A. L. Sonenshein, personal communication) molecules per cell, respectively. Intracellular repressor concentrations – in the case of chromosomally encoded repressors – appear to correlate at least partially with the amount of genes they regulate. The Lac repressor, present in very low concentration, regulates only one operon, while CcpA and CodY are involved in the direct regulation of 100 and 25 genes or operons, respectively (Sonenshein, 2007; Sonnenshein, 2005). Therefore, we wanted to find out if CcpN might regulate more than the three known genes *gapB, pckA* and *sr1*.

A database search reveals 291 potential CcpN targets

Therefore, the genome of *B. subtilis* was searched for possible CcpN binding sites using the SubtiList Web Server (http://genolist.pasteur.fr/SubtiList/) and a slightly revised version of the CcpN consensus sequence (TRTGHYATAYW) reflecting naturally occurring binding sites as well as binding sites found by EMSA (Licht *et al.,* 2005, Servant *et al.,* 2005). Additionally, one mismatch in the consensus sequence was allowed and only sequences within –100 bp or +20 bp relative to the translational start site were considered, since the known CcpN operators are located within this range (Servant *et al*., 2005) and the location of the promoters of many genes is still unknown. 291 putative CcpN binding sites were found, among them 22 that perfectly matched the consensus sequence.

EMSA suggests at least three additional targets of CcpN

Out of all potential CcpN targets found, those encoding proteins involved in carbon catabolism or those whose CcpN operator sequence was matching the consensus binding site were selected for further investigation. EMSAs were performed using fragments carrying the putative CcpN operator in parallel with a fragment carrying the consensus binding site of the *sr1* promoter (Fig. 2). Of 20 investigated operators, only three were bound by CcpN: *thyB*, *gcaD* and *yhaM*. However, binding was less efficient than for the *sr1* operator. Apparent K_D values were 770 nM for *thyB*, 2.9 µM for *yhaM* and 3.4 µM for *gcaD*, compared to 420 nM for the *sr1* single site K_D value. Although the *thyB* operator shows one mismatch to the consensus sequence, its K_D value is still significantly higher than the K_D for *yhaM*, which is almost a perfect consensus sequence.

The *thyB* gene encodes the minor thymidylate synthase of *B. subtilis*, contributing to only 5 % of thymidylate synthesis (Neuhard *et al*., 1978). Interestingly, the main thymidylate synthase of *B. subtilis* is closely related to thymidylate synthases encoded by phages, while *thyB* resembles the thymidylate synthases found in other bacteria (Tam & Borriss, 1998). The *yhaM* gene codes for a 5'-3' exoribonuclease (Oussenko *et al*., 2002) and *gcaD* encodes UDP-N-acetylglucosamine pyrophosphorylase involved in cell-wall buildup (Hove-Jensen, 1992).

Since the K_D values for CcpN in the putative *gcaD* and *yhaM* operators indicated a very weak binding, we focused on *thyB* in further experiments.

DNase I footprinting reveals one large binding site for thyB

All previously investigated CcpN operators have two binding sites, one closely resembling the consensus binding site and one vastly differing from it (Licht & Brantl, 2006; Servant *et al.,* 2005). To determine binding sites at the *thyB* promoter, DNase I protection footprinting was performed (Fig. 3a). A situation resembling the *pckA* promoter was found, with only one long stretch of protected

bases that includes both the –10 and the –35 region. As in *pckA*, the consensus binding site overlapped with the –10 region. The regions protected by CcpN are summarised in Figure 3a.

At the *sr1*, *pckA* and *gapB* promoters, there is one strong and one weak binding site, but these differences are overcome by CcpN binding cooperatively to the two sites, so that both sites are bound with the same efficiency when they are present on one DNA fragment and equally well protected from DNase I (Licht & Brantl, 2005). Interestingly, protection from DNase I was not constant within the protected region at the *thyB* promoter.

To verify that CcpN binds specifically at the *thyB* promoter, a *thyB* fragment carrying a mutation in the CcpN consensus binding site was subjected to DNase I footprinting. Indeed, no footprint was obtained (Fig. 3a).

Chemical interference footprinting identifies bases contacted by CcpN at the thyB *promoter*

Chemical interference footprinting experiments were performed to determine protein-DNA contacts at a higher resolution. Since neither contacts to C residues nor to the sugar-phosphate backbone played a significant role in the previously studied CcpN-operator interactions (Licht & Brantl, 2006) only methylation and $KMnO_4$ interference footprints were performed to detect G and A or T residues contacted by CcpN, respectively.

The *thyB* promoter shows a very unusual contact distribution compared with the previously investigated CcpN operators (Fig. 3b+c). The closest contacts are located within the consensus sequence, but the other contacts are almost evenly distributed over the remaining region protected in DNase I footprinting. A similar DNase I protection pattern was found at the *pckA* promoter, but chemical footprinting showed clearly two distinct binding sites, located two helical turns apart (Licht & Brantl, 2006). At the *thyB* promoter, three binding sites are present in total, one strong and two weaker ones. Since in DNase I footprints primarily the consensus sequence was protected, the other two binding sites might be auxiliary sites serving to guide CcpN to its main binding site – a feature that can also be observed with the Lac repressor (Oehler *et al*., 1990). At the *thyB* promoter, contacts to T's are more prominent than contacts to G's and A's, whereas at the *sr1*, *gapB* and *pckA* promoters Gs were the bases forming the closest contacts with CcpN. Interestingly, the upstream binding site seems to rely mainly on contacts to A's whose modification occurs exclusively in the minor groove indicating that CcpN is able to access this much narrower DNA groove. Minor groove binding is often found in the form of 'indirect readout', like in CRP or HU of *E. coli* (Lindemose *et al*., 2008, Swinger & Rice, 2007) and does not provide much binding specificity due to the low information content. However, it greatly increases binding stability. ComK, the *B. subtilis* competence regulator, was found to specifically bind its operator site through contacts in the minor groove, which leads to a novel potential activation mechanism (Smits *et al*., 2007). It is possible that CcpN exerts the same mechanism at the *thyB* promoter.

A comparison of previously investigated CcpN operators and operator at the *thyB* promoter can be found in table S2.

Reporter gene assays and in vitro *transcription demonstrate that CcpN acts as an activator at the* thyB *promoter*

To assay the *in vivo* relevance of CcpN binding to the *thyB* promoter, transcriptional *thyB-lacZ* fusions were integrated into the chromosome and β-galactosidase activities measured. Strains were grown in glucose-free SP medium and glucose added where appropriate. The comparison between DB104 wild-type and isogenic *ccpN* knockout strain allowed for detection of an influence of CcpN. The results showed a small but significant and reproducible effect of CcpN (Fig. 4a). Whereas transcription is increased by a factor of 1.5 upon glucose addition in the wild-type strain, this effect cannot be observed in the *ccpN* knockout strain, where transcription levels in the absence and presence of glucose are nearly identical. To substantiate these results, a *lacZ*-fusion with a *thyB* promoter carrying a mutation that prevents CcpN-operator-interactions (see Fig. 3a) was constructed and analysed as above. The site and type of mutation was chosen based on previously performed EMSAs (Licht *et al.*, 2005), where the mutated base has been shown to be invariant. This construct did not respond to glucose, indicating that CcpN truly regulates the *thyB* promoter (Fig. 4a). This finding is particularly striking, since CcpN has only been shown to work as a repressor under glycolytic

conditions (Licht *et al*., 2005, Servant *et al.,* 2005), but here, it acts as an activator. A statistical significance test confirmed the difference in activation between wild-type and knockout strain with a confidence of > 98 %. Compared to the huge effects CcpN exerts as a repressor, the relatively small effect observed here might be due to the significantly greater K_D value observed for the *thyB* promoter. Beside CcpN, there are few other examples of DNA-binding proteins that use an identical consensus sequence for both activation and repression. Two prominent examples are the global regulators CodY and CcpA from *B. subtilis* (Henkin, 1996; Sonenshein, 2005), the former clearly resembling the case of CcpN, whereas the latter requires HPr as a co-regulator and acts as activator or repressor depending on operator positions.

To corroborate these findings, *in vitro* transcription experiments with *B. subtilis* RNA polymerase were performed (Fig. 4b). This assay clearly showed that CcpN is able to activate *thyB* transcription under conditions of low pH and high ATP concentration, conditions found to be required for CcpN at the three known target promoters. This is in good agreement with the results of the β-galactosidase measurements, showing that CcpN is able to activate the transcription of *thyB* both *in vivo* and *in vitro*. It remains unclear how CcpN is able to act as an activator upon binding to the -10 region, but other studies have shown the same effects for *B. subtilis* CodY at the *ackA* promoter and the *E. coli* MerR protein (Shivers *et al*., 2006; O'Halloran *et al*., 1989).

Regarding the physiological relevance of CcpN-mediated activation of *thyB transcription*, one could hypothesise that more thymidylate is needed during glycolysis, which usually comes along with excellent growth conditions and increased growth rate. ThyB contributes to only about 5 % of TSase activity in *B. subtilis* (Neuhard *et al*., 1978), and an activity increase of 50 % does not seem relevant in the background of ThyA. However, ThyB might be the "original" TSase of *B. subtilis'* ancestors, and while its activity has been almost lost, the regulatory mechanism is still intact. The results presented here might also indicate that ThyB is involved in the formation of pyrimidine nucleotide activated sugars during glycolysis and therefore upregulated independently of ThyA. Since it is not excluded that CcpN might regulate thymidylate synthetase genes in other Gram-positive bacteria that possess – in contrast to *B. subtilis* - only one *thy* gene, an alignment of CcpN binding sites at *thyB* promoters in related Gram-positive species was performed.

Alignments show potential CcpN binding sites at thyB *promoters in related Gram-positive species*

To investigate if CcpN operator sites are located upstream of *thyB* genes in these species, all bacteria encoding CcpN homologues were scanned for *thyB* related genes using BLAST (Zhang *et al*., 2000). Species with the highest *thyB* homology compared to *B. subtilis* were subsequently searched for CcpN operator sites upstream of the *thyB* start codon, since the transcriptional start sites are not known in most of these organisms. Figure 5 shows the alignments. Interestingly, in *B. amyloliquefaciens*, *B. anthracis*, *B. cereus* and *S. aureaus* as well as in *F. nucleatum* consensus-like CcpN operators are located upstream of the *thyB* genes in approximately the same distance as in *B. subtilis*. In other bacteria, more mismatches were found in the operator sequence and operator spacing compared to *B. subtilis*. However, it is conceivable that other organisms have other requirements for CcpN consensus operator sites, and that these sites bind their corresponding CcpN proteins quite well.

Overall, it appears that CcpN is involved in the regulation of one more *B. subtilis* gene than previously anticipated and upcoming searches might reveal more new targets. At least the high degree of conservation regarding binding sites, even between different organisms, strongly favours this hypothesis.

This report demonstrates that bioinformatics in concert with molecular biological methods can be used to identify new targets of a transcriptional regulator, even if the biological context in which this regulator acts on these targets is not yet understood. In the case of *thyB*, analyses in other firmicutes that have both a CcpN homologue and a CcpN binding site upstream of their *thyB* genes and encode – in contrast to *B. subtilis* – only one thymidylate synthetase, might shed light on a possible role of CcpN-mediated regulation of these genes.

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Figure Legends

Figure 1
Determination of the intracellular concentration of CcpN
Western Blot of a 12 % SDS polyacrylamide gel. 1-3: parallels of protein crude extract of *B. subtilis* DB104, corresponding to 1.2×10^8 cells; 4: 2.2 pmol of purified CcpN-His_5; 5: 4.4 pmol of purified CcpN-His_5; 6: 1.4 pmol of purified CcpN-His_5 in protein crude extract of DB104 (*ccpN*::*cat*); 7: 5 pmol BSA. The blot was developed using horseradish peroxidase catalysed conversion of diaminobenzidine, PC-BAS 2.08e software was used for quantification. M: band corresponding to the protein monomer; D: band corresponding to a putative protein dimer.

Figure 2
EMSA with different putative CcpN targets
EMSAs of double-stranded 23 bp DNA fragments containing the consensus binding site of the *sr1*, *gcaD*, *thyB* and *yhaM* operators. The DNA was incubated with increasing concentrations of purified CcpN-His_5 (CcpN concentration from left to right: 0 nM; 99 nM; 296 nM; 889 nM, 2.67 µM and 8 µM). The autoradiograms of the gels are shown.

Figure 3
DNase I footprinting and interference footprinting of the thyB *promoter*
(a) DNase I footprint. GA + CT: sequencing reaction; -: control without DNase I. The DNA was incubated with increasing concentrations of purified CcpN-His_5 (CcpN concentration from left to right: 0 nM; 296 nM; 889 nM, 2.67 µM and 8 µM) prior to DNase I treatment. The combined protected sites have been designated site I+II. The autoradiograms of the gels are shown. Left, wild-type *thyB* promoter; right, mutated *thyB* promoter. An Overview of the protected region at the *thyB* promoter is shown below the gels. Bases protected by CcpN are coloured with a grey background. –35 and –10 regions, the transcriptional start site, the CcpN-operator consensus and the mutated operator sequence used in *lacZ* fusions (mutation shown in inverted colours) are indicated.
(b) Methylation interference footprint of the *thyB* promoter. CT: Maxam-Gilbert C+T sequencing reaction; C, control (protein-free methylated DNA); B and U, bound and unbound fraction of methylated DNA subjected to binding with CcpN-His_5. Close contacts are indicated by black triangles.
(c) $KMnO_4$ interference footprint of the *thyB* promoter. Abbreviations are as in C. GA: Maxam-Gilbert G>A sequencing reaction; C, control (protein-free $KMnO_4$-treated DNA).

(d) Column diagrams indicating the relative strength of interference signals for both strands of the three operators. Only positive signals, i.e. signals that indicate contacts, are shown. Measured values are averaged from four independent experiments.

Figure 4

A. *β-galactosidase assays*

Column diagrams showing the amount of Miller units measured at the *thyB* (with wild type or mutated CcpN operator) promoter under repressing and non-repressing conditions in the DB104 wild-type and *ccpN* knockout strains. The cells were grown in SP medium till an OD_{600} of 2.0. Values are averaged over 6 different clones and three independent experiments.

B. In vitro *transcription at the* thyB *promoter*

In vitro transcription assay using 100 nM of a DNA fragment and 50 nM of His-tagged *B. subtilis* RNAP as well as 50 nM purified SigA in each reaction. The autoradiogram of the gel is shown. Reaction conditions are denoted above each lane and the *thyB* transcript is indicated by a black arrow. One of three independent experiments is shown. LC, loading control.

Figure 5

Alignment of promoter regions

Alignment showing the regions upstream of the translational start site of *thyB* of different bacteria possessing a *ccpN* homologue or orthologue. The translational start site is indicated in bold and underlined. Sequences resembling the *B. subtilis* consensus binding site for CcpN have been labelled in bold and with black rectangles. Deviations from the consensus sequence are marked by grey letters.

Table 1: Strains and plasmids used in this study

Strain	Genotype	Reference
E. coli K12 DH5α	F- φ80*lac*ZΔM15 Δ(*lacZYA-argF*) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *phoA sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1	Invitrogen™
E. coli BL21 (DE3) (pETSigA)	*dcm ompT hsdS(rB-mB-) gal λ(DE3)*	Fujita *et al.*, 1998
B. subtilis NIG2001	*trpC2 pheA1 neo*^{r} *rpoC-His*$_6$	Fujita *et al.*, 1998
B. subtilis DB104	*his nprR2 nprE18 ΔaprA3*	Kawamura & Doi, 1984
B. subtilis DB104	*his nprR2 nprE18 ΔaprA3* (*ccpN*::*cat*)	Licht *et al.*, 2005

Plasmid	Description	Reference
pAC6	pBR322 based vector for integration of transcriptional *lacZ* fusions into *amyE* locus of *B. subtilis*, Ap^R, Cm^R	Stülke *et al.,* 1997
pTHY1	pAC6 with p_{thyB}-*lacZ* fusion	this work
pATM1	pAC6 with p_{thyB}-*lacZ* fusion with mutated CcpN operator	this work

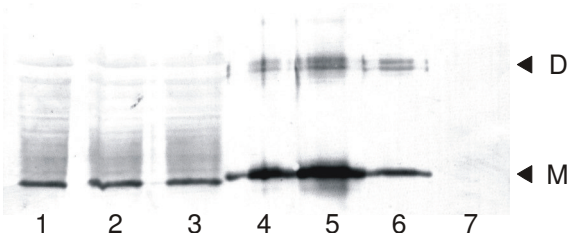

Figure 1 Eckart *et al.*

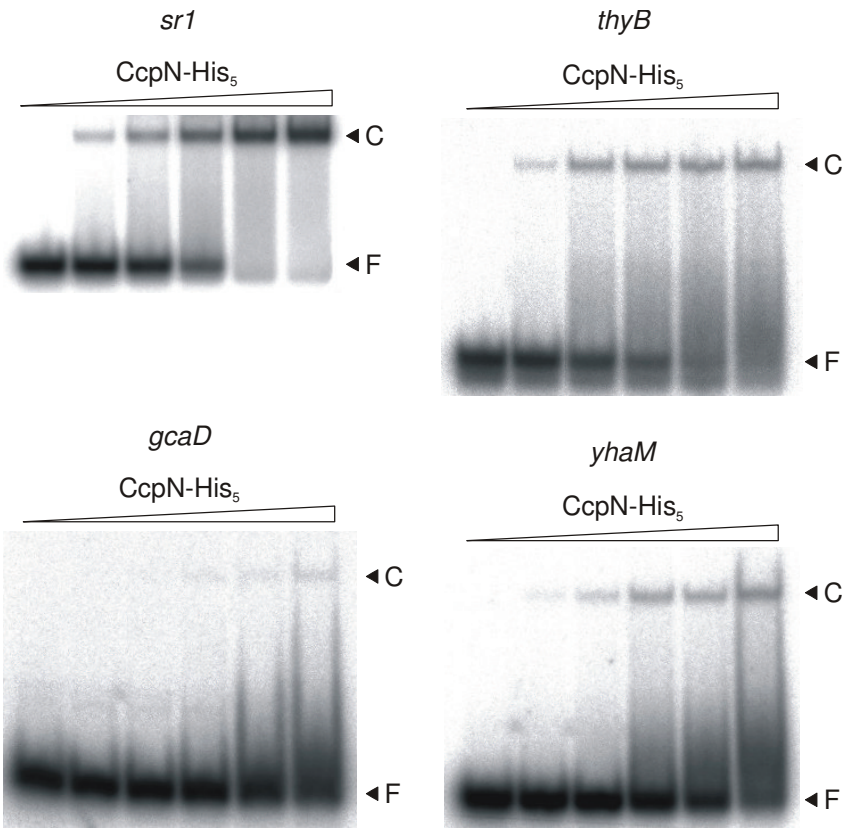

Figure 2 Eckart *et al.*

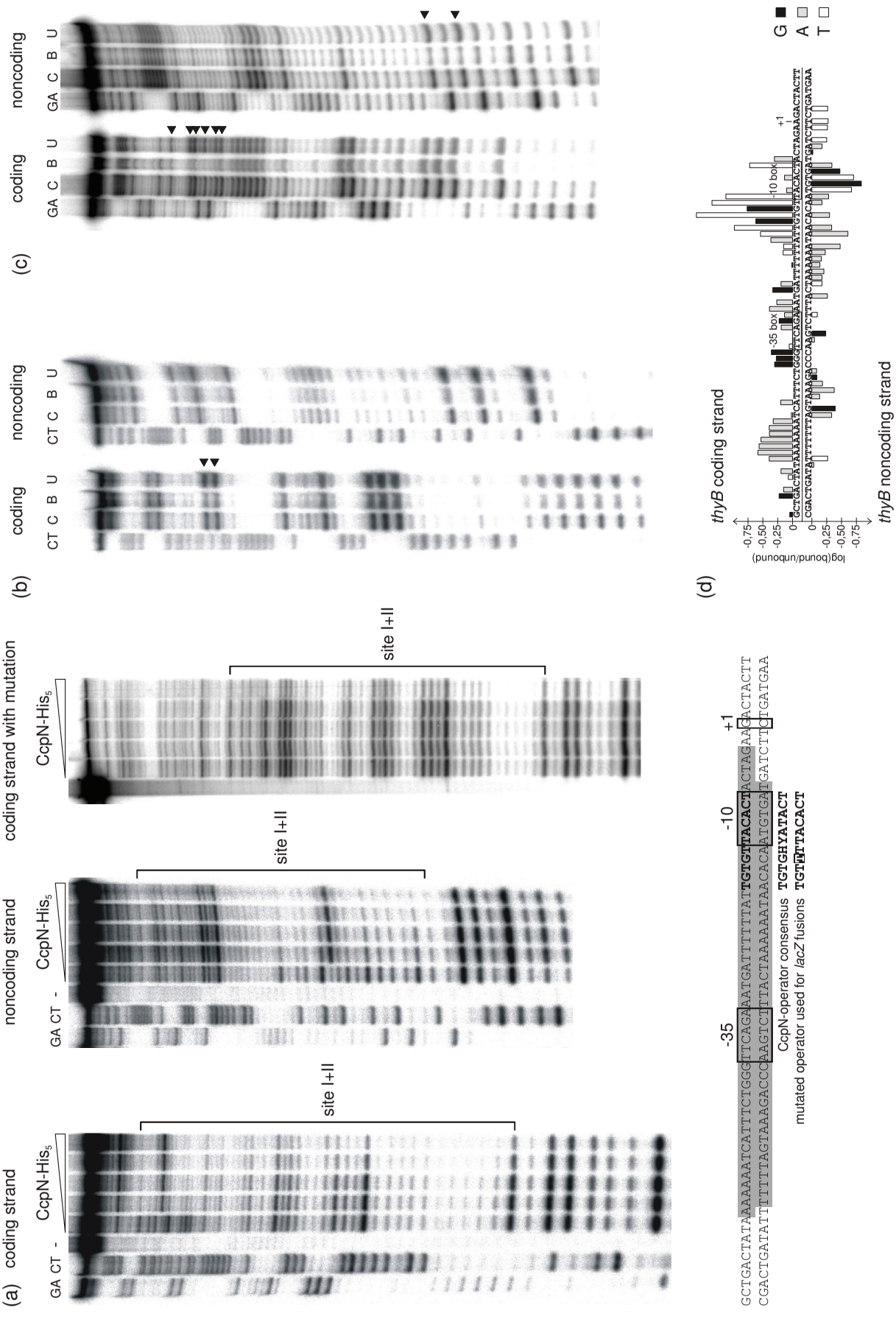

Figure 3 Eckart *et al.*

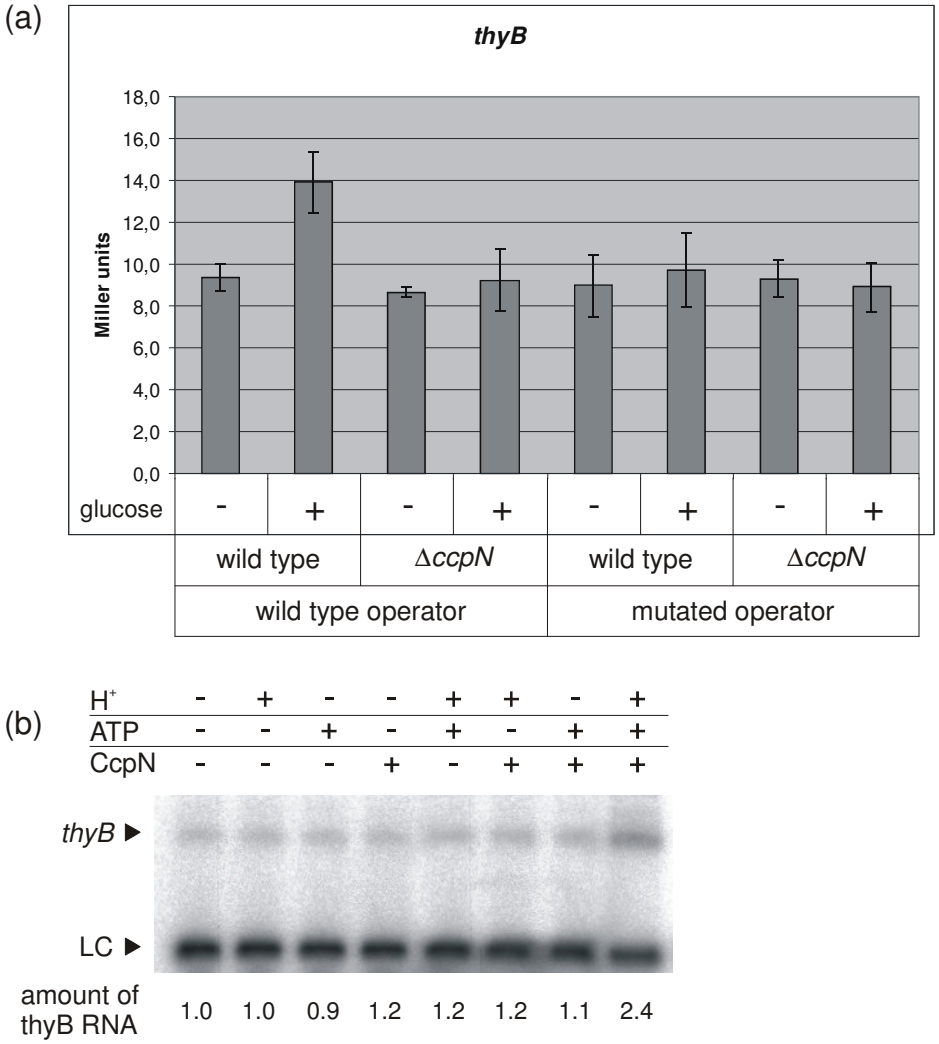

Figure 4 Eckart *et al.*

B. subtilis ATTGTGTTAC ACTACTAGAA GACTACTTTT AAAGGATGAA AAAAATG

B. amyloliquefaciens ATTGTGTTAC ACTACTAAAA GACTACTTTA AAAGGATGAA AAAAATG

B. anthracis GTTATGATAA ATTGCATATA AGATTGAAAG AAGGTTTTAC TACATATG

B. cereus TTTATGATAA AATCCATATA GACTAGAGAT TTGAAAGAAG GTTTTTCTAC ATATG

B. halodurans TGTGCTAGTG AGGATACATA AGCAGATTGG ATTGTGCATA ATATGTACTA
AATGACAATC CTCCATGCAT ATTTTTCGTT TTCGCCGGGT GAACGAAGTC
GCACTGGCGA AAAGGATCAA ATG

F. nucleatum TATGTGGTAT AATAATATAA AATTAAAAAA ATAGGAGAGA GGAAAATG

O. iheyensis TTTATGTTCT AATAGTGAGT TTATGTTTTA AGCTTATGTA ACAAAAGAGG
ACTAGTATCT GTTATAATTA AATCATTTGA GAAGCAAAAT CTATATTTTA
AATATAGTCA GGAGAGATTA ATATATG

S. aureus ATTATGTCGA ACTAAATATA CATACTATAA ATAATGAAAA TGAGGTGTTA TCGCATATG

S. epidermitis TATATGAGAT ATTTTACATA AAAGGTTTAA AATTTTATAA ACTCAAAACC
CTCTCTCTTC TATCGATACA TCTTTTCTAG TATAATGTTT ATTAATATAA
TACATATTTT TATAAGGAGT TGTACGCATG

S. agalactiae TTTTTGCTAA ATTCCATTTT ACCATAAAGA AGCTAAAAAT ATGAAAAAAA
GCTTTAACCT TCAAAGTCTT GTTTCTCACT AGAATATCTT TTTAAAATCT
GATAAAATAA GACTTTGGAG GTATCTCATG

Figure 5 Eckart *et al.*

7. Diskussion

Bacillus subtilis besitzt ein ausgefeiltes Regulationssystem, um auf Änderungen des Nährstoffangebots angemessen reagieren zu können. Der Hauptakteur dieses Katabolitrepressionssystems ist CcpA, das unter Mitwirkung von HPr ca. 100 Gene reguliert und bioinformatischen Analysen zufolge noch 200 weitere regulieren könnte. Daneben gibt es noch weitere, jedoch nicht weniger bedeutsame Transkriptionsfaktoren, die an der Katabolitrepression beteiligt sind, wie z. B. CcpC, CggR oder den kürzlich identifizierten Transkriptionsfaktor CcpN. Letzterer reguliert die Transkription der ausschließlich während der Gluconeogenese aktiven Gene *pckA* und *gapB* sowie der kleinen regulatorischen RNA SR1. In ersten Arbeiten (Servant *et al.*, 2005; Licht *et al.*, 2005) wurde CcpN nur ansatzweise charakterisiert. Mittels EMSAs konnte eine spezifische Bindung an die Promotorregionen der regulierten Gene gezeigt werden, und DNase-I-Footprints lieferten eine grobe Position der Operatoren. Später wurden die biophysikalischen Parameter der CcpN-DNA-Interaktion aufgeklärt (Zorrilla *et al.*, 2008) sowie die von CcpN beeinflussten metabolischen Flüsse charakterisiert (Tännler *et al.*, 2008). Im Rahmen der vorliegenden Arbeit wurde die Interaktion zwischen CcpN und der DNA im Detail untersucht, die Sequenzvoraussetzungen der Operatoren verifiziert sowie die intrazellulären Regulatoren von CcpN bestimmt. Außerdem wurde der Repressionsmechanismus von CcpN aufgeklärt und ein potentielles neues Zielgen von CcpN identifiziert.

7.1. CcpN-DNA-Interaktionsanalyse

7.1.1. Bindungsmuster und Operatorverteilung von CcpN

In dieser Arbeit wurde eine hochauflösende Bestimmung der für eine CcpN-Bindung nötigen Basen mittels Interferenz-Footprinting an den einzelnen Operatoren durchgeführt. Dabei stellte sich heraus, dass CcpN an allen drei untersuchten Promotoren zwei Bindungsstellen besetzt, von denen jeweils eine stärker als die andere kontaktiert wurde. Es konnte auch für den *pckA*-Operator, der im DNase I-Footprint nur eine ausgedehnte Bindungsstelle aufwies (Servant *et al.*, 2005), gezeigt werden, dass zwei deutlich voneinander getrennte Operatoren existieren. Allen Operatoren an den drei Promotoren ist gemein, dass die am intensivsten kontaktierten Basen Guanin und Thymin sind. Zu Adenin-Resten wurden mit einigen Ausnahmen in der Mehrzahl nur schwächere Kontakte detektiert, zu Cytosin-

Resten durchweg nur wenige und sehr schwache Kontakte. Im Gegensatz zu Repressoren wie CopR, die ihre Operatoren an nur wenigen Basen kontaktieren (Steinmetzer *et al.*, 1997) bildet CcpN extensive Kontakte zu fast allen Basen im Operator aus, eine Eigenschaft, die z. B. auch der Transkriptionsfaktor TyrR aus *E. coli* aufweist (Hwang *et al.*, 1999). Wie auch im Fall von TyrR gefunden, existieren auch nur marginale Kontakte zwischen CcpN und dem Zucker-Phosphat-Rückgrat der DNA, was den Beitrag dieser Interaktion für die Bindung vernachlässigbar macht. Offensichtlich erlauben vielfältige und starke Kontakte zu den Basen, auf eine Kontaktierung des DNA-Rückgrats zu verzichten. Die starke Kontaktierung von Guanosin-Resten zeigt, dass CcpN intensive Kontakte zur großen Furche der DNA ausbildet, da die Methylierung von Guanosin am N7-Atom stattfindet, welches in die große Furche weist. Kontakte zur großen Furche findet man bei vielen Transkriptionsfaktoren, wie z. B. RhaS aus *E. coli* (Bhende & Egan, 1999), da der Informationsgehalt in der großen Furche deutlich höher als in der kleinen ist. Die schwache Kontaktierung von Adenin-Resten zeigt, dass CcpN auch Interaktionen mit Basen in der kleinen Furche ausbildet, da die Methylierung von Adenin am in die kleine Furche weisenden N3-Atom stattfindet. Aufgrund des geringen Informationsgehaltes ist denkbar, dass diese Interaktionen neben der Sequenzerkennung durch das Fehlen von Kontakten zum DNA-Rückgrat auch oder vor allem der Stabilisierung der CcpN-DNA-Interaktion dienen.

Betrachtet man alle Kontakte, stellt man fest, dass diese sich im Fall der starken Bindungsstellen auf einen definierten Kernbereich an jedem Operator konzentrieren, der sehr gut mit der vorher für CcpN ermittelten Konsensussequenz übereinstimmt. Am *sr1*-Operator wurden zwei annähernd gleich stark kontaktierte Operatoren festgestellt, wobei die upstream liegende Bindungsstelle geringfügig stärker kontaktiert wurde. Auch entsprechen beide Bindungsstellen mit jeweils einer Abweichung annähernd der Konsensussequenz. Ein völlig anderes Bild bieten der *pckA*- und der *gapB*-Promotor. Hier sind fast alle Kontakte auf eine Bindungsstelle beschränkt, die zweite Bindungsstelle wird nur äußerst schwach und an wenigen Basen kontaktiert und weist zudem zahlreiche Abweichungen von der Konsensussequenz auf. Transkriptionsfaktoren, die ihre Operatoren derart unterschiedlich stark kontaktieren, sind eher selten. Die meisten Transkriptionsfaktoren binden ihre Operatoren mit mehr oder weniger gleicher Intensität (Brenowitz *et al.*, 1986; Steinmetzer & Brantl, 1997; Lewis, 2005). Allerdings gibt es auch Gegenbeispiele, wie z. B. den PurR-Repressor aus *B. subtilis* (Shin *et al.*, 1997, Bera *et al.*, 2003), auch wenn hier die Unterschiede nicht so stark ausgeprägt sind wie im Falle von CcpN.

Die vorgestellten Ergebnisse erlauben auch eine Erklärung der Effekte, die von Servant *et al.* bei EMSAs mit mutierten *gapB*-Promotorfragmenten beobachtet wurden. Zwei der getesteten Mutationen, T(–11)→A und A(23)→G resultierten in einer deutlich schwächeren CcpN-Bindung und können damit erklärt werden, dass sie Basen betreffen, die bei der Bestimmung der Konsensussequenz als invariant ermittelt wurden und außerdem intensive Kontakte zu CcpN ausbilden.

Interessant ist auch die Verteilung der Operatoren an den drei Promotoren: Während beim *sr1*-Promotor der starke Operator upstream der –35-Box mit dem Zentrum bei –48 und der schwächere zwischen der –35- und der –10-Box liegt, wobei sich ein schwacher Kontakt in der –10-Box befindet, ist die Operatorverteilung an den beiden anderen Promotoren davon deutlich verschieden. Sowohl am *pckA*- als auch am *gapB*-Promotor überlappt die starke Bindungsstelle komplett mit der –10-Box. Die schwache Bindungsstelle bedeckt beim *pckA*-Promotor die –35-Box, während sie beim *gapB*-Operator an Position +19 lokalisiert ist. Einige Transkriptionsfaktoren weisen im Gegensatz zu CcpN eine hochkonservierte Operatorverteilung auf, wie z. B. CytR aus *E. coli* (Collado-Vides *et al.*, 1991). Allerdings ist eine unterschiedliche Verteilung von Operatoren des gleichen Repressors an verschiedenen Promotoren nicht ungewöhnlich. Ein Beispiel dafür ist CcpA aus *B. subtilis*, dessen Operatoren sich z. B. an den Positionen –33, –3 oder +37 befinden können (Weickert & Chambliss, 1990; Grundy *et al.*, 1994). CcpA kann an allen diesen Operatoren als Repressor wirken, wobei je nach Operatorposition der Repressionsmechanismus variieren kann. Eine Übersicht über alle Kontakte an den drei untersuchten Promotoren ist in Abbildung 5 dargestellt.

7.1.2. Kooperative Bindung von CcpN

Die Interferenz-Footprinting-Experimente zeigten deutlich, dass die beiden CcpN-Operatoren an einem Promotor unterschiedlich stark kontaktiert werden. In EMSAs mit Oligonucleotiden, die jeweils nur einen Operator trugen, konnten diese Befunde bestätigt werden. Dabei entsprechen die ermittelten K_D-Werte qualitativ der beobachteten Kontaktintensität: Die Operatoren des *sr1*-Promotors, die sich nur leicht in den Kontakten unterscheiden und beide nahe an der Konsensussequenz sind, binden CcpN mit ähnlicher Affinität und einem K_D-Wert von 420 nM für den starken und 650 nM für den schwachen Operator. Beim *pckA*-Operator wird die stark vom Konsensus abweichende Bindungsstelle deutlich schlechter (K_D = 2,53 µM) als der starke Operator (K_D = 290 nM) gebunden.

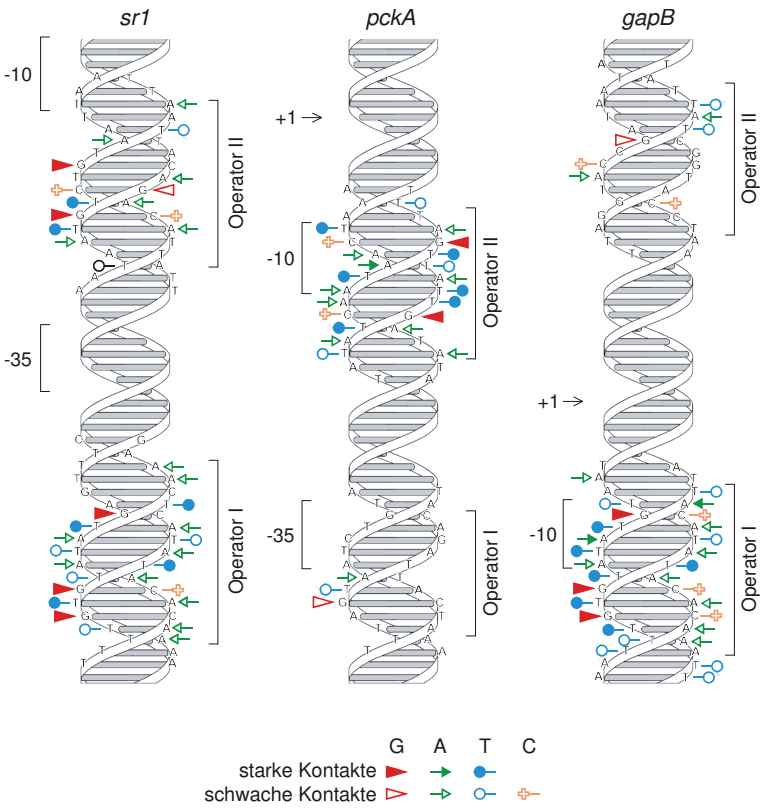

Abbildung 5: Übersicht über CcpN-DNA-Kontakte

Übersicht über signifikante Kontakte an den drei CcpN-regulierten Promotoren. Gefüllte Symbole stellen starke Kontakte dar (50-100 % Signalstärke verglichen mit dem stärksten Signal), leere Symbole schwache Kontakte (15-49 % Signalstärke). Kontakte mit weniger als 15 % relativer Kontaktstärke sind nicht gezeigt. Die Position der –10-Box, der –35-Box, des Transkriptionsstarts und aller Operatoren sind angegeben.

Besonders drastisch fällt der Effekt am *gapB*-Promotor aus. Hier bindet der starke Operator, der eine perfekte Konsensus-Sequenz darstellt, CcpN mit einem K_D-Wert von 98 nM, der schwache hingegen nur mit einem K_D-Wert von 4,4 µM. Befinden sich allerdings beide Operatoren auf einem DNA-Fragment, gleichen sich die K_D-Werte für die Einzeloperatoren einander an. Während die Bindungsstellen des *sr1*-Promotors beide etwas fester gebunden werden (K_D = 80 nM für beide Operatoren), zeigten am *pckA*- und *gapB*-Promotor vor allem die schwachen Bindungsstellen eine deutliche Zunahme der Bindungsstärke auf einen K_D-Wert von 145 nM beim *pckA*-Promotor und 114 nM beim *gapB*-Promotor. Diese Zunahme an Bindungsaffinität ergibt einen Energiegewinn von ca. 10 kJ/mol an allen drei Promotoren, wenn beide Bindungsstellen in relativer räumlicher Nähe auf einem DNA-Fragment vorliegen. Dies und eine Veränderung in Form und Anstieg der Bindungskurve, die aus einem Hill-Koeffizienten >1 resultiert, zeigen deutlich, dass die CcpN-Operatoren in einer kooperativen Art und Weise besetzt werden. Diese Kooperativität äußert sich allerdings auf verschiedene Art an den drei Promotoren. Während der *sr1*-Promotor aus zwei annähernd gleichen Operatoren besteht, die bei Vorhandensein auf einem DNA-Fragment beide intensiver gebunden werden, sind der *pckA*- und der *gapB*-Promotor aus einem Haupt- und einem Hilfsoperator zusammengesetzt. Die Affinität des Hauptoperators bleibt dabei konstant, allerdings wird die Affinität des Hilfsoperators in Gegenwart des Hauptoperators drastisch

erhöht. Unterschiedlich starke Bindungsstellen wurden z. B. auch beim DeoR-Repressor aus *B. subtilis* beobachtet (Zeng & Saxild, 1999). Dessen Operator besteht aus einer kompletten und einer halben Bindungsstelle, allerdings bindet DeoR im Gegensatz zu CcpN keine einzelnen Bindungsstellen.

Interessanterweise liegen die Operatoren für CcpN an den drei Promotoren immer ein ganzzahliges Vielfaches einer Helixwindung auseinander, zwei am *pckA*-Promotor und drei am *sr1*- und *gapB*-Promotor. Da die kooperative Bindung von CcpN eine Interaktion der an den einzelnen Operatoren gebundenen Proteine nahelegt, ist es wahrscheinlich, dass am *sr1*- und am *gapB*-Promotor eine Biegung der DNA stattfindet, um die beiden Repressoren in räumliche Nähe zueinander zu bringen.

7.1.3. Energetische Betrachtung der CcpN-DNA-Interaktion

Die Bestimmung der Bindungskonstanten und daraus der freien Bindungsenergie bei verschiedenen Temperaturen zeigten, dass die Bindung von CcpN an seine Operatoren ein entropisch ungünstiger Prozess ist. Nur durch eine deutlich höhere Bindungsenthalpie, die hier vor allem durch die zahlreichen Kontakte zu Basen der DNA entsteht, wird der ungünstige entropische Beitrag überwunden und eine stabile Interaktion zwischen CcpN und seinen Operatoren ermöglicht. Diese Tatsache hat eine praktische Bedeutung für *B. subtilis*: Da dieses Bakterium Temperaturen von 12 °C bis 52 °C toleriert, sorgen eine hohe temperaturunabhängige Bindungsenthalpie und eine niedrige temperaturabhängige Entropieänderung dafür, dass CcpN, unabhängig von der jeweiligen Umgebungstemperatur, immer gleich stark an seine Operatoren binden und seine Aufgabe erfüllen kann.

7.2. Identifizierung der Regulatoren von CcpN

7.2.1. Aktivierung der CcpN-Aktivität

Bereits eine erste Charakterisierung des *ccpN*-Gens zeigte, dass dessen Expression nicht reguliert ist (Servant *et al.*, 2005), was zu einer konstanten CcpN-Konzentration in der Zelle führt. Da die CcpN-vermittelte Repression allerdings nur unter glycolytischen Bedingungen erfolgen darf, ist ein Regulator für CcpN erforderlich, der dessen Aktivität moduliert. In dieser Arbeit wird die Suche und Identifizierung intrazellulärer Modulatoren der CcpN-Aktivität beschrieben. Mit Hilfe von *in vitro*-Transkriptions-Assays wurde gezeigt, dass CcpN

ohne zusätzliche Faktoren in sehr hohen Konzentrationen spezifisch die Transkription an den kontrollierten Promotoren reprimieren kann, während Kontroll-Promotoren nicht betroffen waren. EMSAs, die mit Protein-Rohextrakten aus *B. subtilis* durchgeführt wurden und in denen signifikante Mengen an Protein-DNA-Komplex detektiert werden konnten (Licht *et al.*, 2005) liefern einen Hinweis darauf, dass die CcpN-Konzentration in der Zelle relativ hoch sein muss. Dies und die beobachtete Modulator-unabhängige Repression erlauben es, die starke Derepression des *pckA*- und *gapB*-Gens zu erklären, die von Servant *et al.* in einem *ccpN*-Knockout-Stamm beobachtet wurden und liefern zudem einen Hinweis darauf, dass CcpN auch *in vivo* ständig an seine Operatoren gebunden ist. Transkriptionsfaktoren, die ihre Operatoren konstitutiv besetzen, sind nicht ungewöhnlich. Ähnliche Beobachtungen wurden auch für das ResD-Protein aus *B. subtilis* (Härtig *et al.*, 2004), das das *yclJK*-Operon bei Sauerstoff-Mangel induziert oder das NorR-Protein aus *E. coli* berichtet (Tucker *et al.*, 2006). Allerdings üben diese beiden Proteine, im Gegensatz zu CcpN, keine konstitutive Repression an den von ihnen regulierten Promotoren aus. Eine geringe Derepression (1,3-fach) in einem Repressor-Knockout-Stamm wird häufig berichtet, wie z. B. beim BzdR-Repressor aus *Azoarcus* sp. CIB (Barragá *et al.*, 2005), allerdings ist eine derart starke konstitutive Repression, wie sie bei CcpN beobachtet wurde (5,4-fach) sehr ungewöhnlich und ihre physiologische Relevanz beim derzeitigen Erkenntnisstand nicht erklärbar.

Um einen Hinweis auf den Regulator von CcpN zu bekommen, wurden β-Galactosidase-Aktivitätsbestimmungen der *sr1*-Promotors in Stämmen durchgeführt, bei denen definierte Schritte der Glycolyse blockiert wurden. Während ein Stamm mit einem Defekt im *gapA*-Gen, das die Glycerinaldehyd-3-Phosphat-Dehydrogenase A codiert (Fillinger *et al.*, 2000), eine gegenüber dem Wildtyp nur marginal verringerte Repression zeigte, war die Repression durch CcpN in einem Stamm mit einem mutierten Glucose-6-Phosphat-Isomerase-Gen um den Faktor 10 geringer (Stülke *et al.*, 2001). Dies legte den Schluss nahe, dass eines der Glycolyseintermediate, die zwischen Glucose-6-Phosphat und 1,1-Bisphosphoglycerat liegen, der gesuchte Regulator für CcpN sein könnte. Allerdings zeigte keines dieser Intermediate einen Einfluss auf die CcpN-vermittelte Repression in *in vitro*-Transkriptions-Versuchen.

Durch eine Domänenanalyse der CcpN-Sequenz wurde festgestellt, dass CcpN zwei CBS-Domänen besitzt. Für diese nach der Cystathion-β-Synthetase benannten Domänen konnte die Bindung des Adenin-Restes verschiedener Nucleotide und Nucleoside nachgewiesen werden (Scott *et al.*, 2004). Eine Untersuchung des Einflusses von ATP auf die Repressionseffizienz von CcpN *in vitro* zeigte zwar eine generelle und unspezifische Verbesserung der Transkriptionseffizienz, jedoch nur einen äußerst geringen spezifischen Effekt auf die

Repression, der die effiziente durch CcpN *in vivo* vermittelte Repression nicht erklären kann. Dies legt den Schluss nahe, dass CcpN noch durch einen zweiten Regulator moduliert wird. Die Erhöhung der Repressionsaktivität von CcpN, die in Gegenwart von saurem, jedoch nicht von neutralem Glycerinaldehyd-3-Phosphat beobachtet wurde, half, den zweiten Regulator von CcpN zu identifizieren: Einen leicht sauren pH-Wert. Zwar ist von zahlreichen Proteinen und Enzymen wie Proteasen und Ionentransportern bekannt, dass ein korrekter pH-Wert für ihre Funktion unerlässlich ist (St. Leger *et al.*, 1998; Laloknam *et al.*, 2006), allerdings gibt es bisher nur einen Transkriptionsfaktor, bei dem eine pH-Sensitivität gezeigt werden konnte: NikR, ein Nickel-bindender Transkriptionsfaktor aus *E. coli*, weist – abhängig vom pH-Wert in der Zelle – eine unterschiedliche Sensitivität gegenüber Nickel-Ionen auf (Fauquant *et al.*, 2006). Die ermittelten Regulatoren fügen sich gut in die Stoffwechselsituation in glycolytischen *B. subtilis*-Kulturen ein: Bei ausreichender Glucoseversorgung wird durch die Glycolyse schnell viel ATP produziert, was einen hohen ATP-Spiegel in der Zelle erzeugt. Durch die Abschaltung des Citratzyklus sammelt sich Acetat als Endprodukt der Weiterverwertung von Acetyl-CoA an, welches zwar später ausgeschieden wird, aber kurzzeitig für eine geringfügige Senkung des zytoplasmatischen pH-Wertes sorgen kann (Tobisch *et al.*, 1999). Allerdings kann damit nicht die Beobachtung einer reduzierten CcpN-vermittelten Repression im Glucose-6-Phosphat-Isomerase-Knockout-Stamm erklärt werden. Da allerdings keine der getesteten in Frage kommenden Substanzen einen Effekt auf die *in vitro*-Transkription hatte, kann es sich bei den Beobachtungen entweder um ein durch den Knockout entstandenes Artefakt handeln, oder es existiert ein bisher unbekanntes Protein, welches den regulatorischen Effekt einer dieser Substanzen *in vivo* vermittelt.

Unter Verwendung von CD-Messungen konnten die durch *in vitro*-Transkription ermittelten Ergebnisse bestätigt werden. Dabei wurde deutlich, dass ATP im leicht sauren Milieu in der Lage ist, deutliche strukturelle Veränderungen in CcpN zu induzieren, allerdings nicht bei neutralem pH-Wert, wo die Veränderungen deutlich geringer ausfielen. Daraus lässt sich ableiten, dass die Bindung von ATP an CcpN zu einem „induced fit"-Mechanismus führt, der häufig bei regulatorischen Proteinen beobachtet werden kann. Beispiele hierfür sind zum einen der Repressor QacR aus *Staphylococcus aureus*, der verschiedene Substanzen binden kann (Schumacher *et al.*, 2004), zum anderen viele metabolische Enzyme oder auch die humane Monoamin-Oxidase, bei der Strukturänderungen durch Ligandbindung ebenfalls durch CD-Messungen nachgewiesen werden konnten (Ramsay *et al.*, 2005).

7.2.2. Repression der CcpN-Aktivität

Es wurde gezeigt, dass ADP in äquimolaren Konzentrationen in der Lage ist, dem Effekt von ATP entgegenzuwirken. Dies erscheint einleuchtend, da zum einen CBS-Domänen neben ATP auch in der Lage sind, ADP zu binden, (Scott *et al.*, 2004), zum anderen festgestellt wurde, dass die ADP-Konzentration in Zellen, die in die Stationärphase eintreten, die ATP-Konzentration signifikant übersteigt (Soga *et al.*, 2002; Soga *et al.*, 2003). Des Weiteren nimmt die ATP-Konzentration in *B. subtilis*-Zellen beim Auftreten von Glucosemangel rapide ab, was letztendlich zur Aktivierung des RsbW/RsbV-Systems der zellulären Stressantwort führt (Voelker *et al.*, 1995; Maul *et al.*, 1995). Die Ergebnisse der CD-Messungen helfen, die Wirkungsweise von ADP zu erklären. Sie zeigen deutlich, dass die drastischen strukturellen Veränderungen in CcpN, die bei einer ATP-Bindung im Sauren zu beobachten waren, bei der Bindung von ADP nicht auftreten. Offensichtlich kehrt CcpN nach der Verdrängung von ATP durch ADP in seinen nicht-repressionsaktiven Zustand zurück. Diese Art der Gegenregulation kann häufig bei Enzymen beobachtet werden, die in Gegenwart bestimmter Signalmoleküle oder Metaboliten an- oder abgeschaltet werden müssen, wie z. B. der Aspartat-Transcarbamoylase aus *E. coli*, die in Gegenwart von ATP aktiviert und in Gegenwart von CTP gehemmt wird (Stevens & Lipscomb, 1990). Bei Transkriptionsfaktoren hingegen ist diese Art der Regulation eher die Ausnahme. Die meisten Aktivatoren oder Repressoren besitzen einen einzelnen Liganden, der – einem An/Aus-Schalter gleich – den Transkriptionsfaktor aktiviert oder inaktiviert. Als Beispiel hierfür sei BzdR und sein Ligand Benzoyl-CoA aus *Azoarcus* sp. CIB genannt (Barragá *et al.*, 2005). Allerdings existieren auch Beispiele von Transkriptionsfaktoren mit mehreren Liganden, wie z. B. GltC aus *B. subtilis*, welches in Gegenwart von α-Ketoglutarat aktiviert und in Gegenwart von Glutamat reprimiert wird (Picossi *et al.*, 2007).

7.2.3. Untersuchung einer CcpN-Mutante

Um diese Befunde zu untermauern, wurde ein CcpN-Protein mit einer Mutation in einem konservierten Rest innerhalb einer der CBS-Domänen untersucht. Aymerich und Declerck haben beobachtet, dass dieses Protein *in vivo* nicht mehr aktiv ist, aber dieselbe Struktur wie natives CcpN besitzt (Chaix *et al.*, Manuskript in Vorbereitung). Die hier durchgeführten *in vitro*-Transkriptionen, in denen das Protein wie der Wildtyp eine effektorunabhängige Repression vermitteln kann, bestätigen diesen Befund und zeigen außerdem, dass die DNA-

Bindungsfähigkeit durch die Mutation nicht beeinträchtigt wurde. Interessanterweise reagiert diese Mutante nicht mehr auf ATP in *in vitro*-Transkriptions-Versuchen, und Aymerich und Declerck konnten außerdem zeigen, dass diese Mutante kein ATP oder ADP mehr binden kann. Damit wurde zum einen ein für die ATP-Bindung wichtiger Rest in CcpN identifiziert und außerdem gezeigt, dass eine direkte ATP-Bindung durch CcpN für eine effiziente Repression essentiell ist.

Zusammenfassend lässt sich feststellen, dass ATP und niedriger pH-Wert die intrazellulären Signale sind, die die Repressionseffizienz von CcpN steuern. Durch EMSAs konnte in dieser Arbeit gezeigt werden, dass diese Effektoren die Affinität von CcpN zur DNA allerdings nicht verändern, was darauf schließen lässt, dass CcpN seine Operatoren *in vivo* ständig besetzt und nur durch eine induzierte Strukturänderung als Metabolismus-spezifischer Repressor aktiv werden kann. Diese Eigenschaft findet man auch bei anderen Transkriptionsfaktoren, so z. B. bei NorR aus *E. coli* (Tucker *et al.*, 2006) oder ResD aus *B. subtilis*, welches ständig an seinen Operator gebunden ist und erst durch eine Phosphorylierung als Repressor aktiv wird (Härtig *et al.*, 2004).

7.3. Aufklärung des Repressionsmechanismus von CcpN

7.3.1. Repressionsmechanismus von CcpN

Obwohl bereits viele Aspekte des Transkriptionsrepressors CcpN erforscht wurden, ist bisher noch nichts über den eigentlichen Repressionsmechanismus bekannt. In dieser Arbeit sollen die Mechanismen, mit Hilfe derer CcpN Repression vermitteln kann, aufgeklärt werden. Durch EMSAs wurde gezeigt, dass CcpN die Bindung der RNAP an den Promotor nicht verhindern kann und deshalb an keinem der drei Operatoren als Inhibitor der Bildung des geschlossenen Komplexes wirkt, da der CcpN-RNAP-DNA-Komplex an allen drei Promotoren unter nicht-reprimierenden und reprimierenden Bedingungen gleich intensiv ist. Als interessant hervorzuheben ist allerdings, dass eine gewisse Konkurrenz zwischen CcpN und der RNAP bezüglich der Promotorbindung besteht. Es wurde gezeigt, dass die Intensitäten aller Protein-DNA-Komplexe im EMSA deutlich abnahmen, sobald sowohl CcpN als auch die RNAP im Ansatz vorhanden waren. Da die Konzentrationen von CcpN und der RNAP so gewählt wurden, dass sie den Konzentrationen *in vivo* entsprechen (Wagner, 2000; Manuskript IV) kann davon ausgegangen werden, dass auch *in vivo* eine Konkurrenz bei der Bindung CcpN-regulierter Promotoren besteht. Dies erklärt auch die

beobachtete starke Derepression von *pckA* und *gapB* in einem *ccpN*-Knockout-Stamm (Servant *et al.*, 2005).

Repressoren, die gleichzeitig mit der RNAP an einen Promotor binden können, reprimieren oft die Bildung des offenen Komplexes, also das Entwinden der DNA-Helix. Beispiele dafür sind der Repressor MerR aus *E. coli* am *merT*-Promotor (Heltzel *et al.*, 1990; Summers, 1992) oder das Protein KorB des Plasmids RK2 (Williams *et al.*, 1993). Während der Operator von MerR mit dem der RNAP überlappt, ist dies bei KorB nicht der Fall. Beide Situationen finden sich auch an den CcpN-regulierten Promotoren, allerdings konnte durch die „Open-complex-formation-Assays" ausgeschlossen werden, dass die Bildung des offenen Komplexes an den drei Promotoren beeinträchtigt ist.

Es konnte gezeigt werden, dass die Synthese abortiver Transkripte am *gapB*-Promotor nicht mehr stattfindet. Dieser Repressionsmechanismus wird von Transkriptionsfaktoren eher selten genutzt. Beispiele dafür sind die *E. coli*-Proteine FIS am *gyrB*- und H-NS am *rrnB*-P1-Promotor (Schneider *et al.*, 1999; Schröder & Wagner, 2000). Für H-NS konnte ein ähnliches Bindungsmuster wie bei CcpN gezeigt werden, bei dem der Operator mit der RNAP-Bindungsstelle überlappt. H-NS ist hier in der Lage, die DNA-Struktur derart zu verändern, dass zwar noch offene Komplexe gebildet werden können, aber eine Initiation der Transkription nicht mehr möglich ist. Ein ähnlicher Mechanismus wäre auch am *gapB*-Promotor denkbar, da hier bei DNase-I-Footprints zahlreiche hypersensitive Stellen durch die Bindung von CcpN induziert werden. Diese Stellen sind in der Regel ein guter Indikator für eine Änderung der DNA-Struktur, was diese Hypothese unterstützt.

Am *pckA*- und *sr1*-Promotor hingegen konnte die Synthese abortiver Transkripte auch unter reprimierenden Bedingungen detektiert werden, allerdings wird der Übergang in einen produktiven Elongationskomplex, also das Verlassen des Promotors durch die RNAP, an diesen Promotoren inhibiert. Dies kann durch zwei mögliche Mechanismen geschehen. Die erste Möglichkeit wäre ein Repressor, der downstream der RNAP bindet und so eine physische Blockade darstellt. Die H-NS-vermittelte Repression des *eltAB*-Operons in *E. coli* funktioniert beispielsweise nach diesem Prinzip (Yang *et al.*, 2005). Betrachtet man allerdings die Position der Operatoren am *pckA*- und *sr1*-Promotor, erscheint diese Art der Repression sehr unwahrscheinlich, was wiederum für die zweite Möglichkeit spricht: Die direkte Interaktion zwischen dem Repressor und der RNA-Polymerase. Es ist bekannt, dass Promotorsequenzen, die in allen Elementen nahezu dem Konsensus entsprechen, den Übergang in einen Elongationskomplex durch eine zu feste Bindung der RNAP verhindern können. Repressoren, die ihre Operatoren in der Regel mit hoher Affinität binden, können

durch eine Interaktion mit der RNAP diesen Effekt nachahmen und die RNAP so am Promotor arretieren. Beispiele dafür sind das Protein p4 des Phagen Φ29 am A2c-Promotor oder auch der Gal-Repressor (Choy *et al.*, 1995; Monsalve *et al.*, 1996).

7.3.2. CcpN-RNAP-Interaktion

Durch Far-Western-Blots und Co-Elutions-Versuche konnte gezeigt werden, dass CcpN in der Lage ist, mit der α-Untereinheit der RNAP, jedoch nicht mit dem σ-Faktor zu interagieren. Betrachtet man dazu die Position der Operatoren am *sr1*- und *pckA*-Promotor, stellt man fest, dass sich diese in einer Position befinden, die CcpN in eine räumliche Nähe zur C-terminalen Domäne der α-Untereinheit bringen kann. Da der Linker der α-Untereinheit die Positionierung der α-CTD flexibel gestaltet, was sich auch an der variablen Position von UP-Elementen in *B. subtilis* zeigt (Meijer & Salas, 2004), ist auch die Positionierung der CcpN-Operatoren flexibel, wie man an den Unterschieden zwischen dem *sr1*- und *pckA*-Promotor sehen kann. Aus diesen Beobachtungen lässt sich schlussfolgern, dass CcpN am *pckA*- und *sr1*-Promotor nach dem oben erwähnten Mechanismus arbeitet und die RNAP durch direkte Interaktion mit der α-CTD am Promotor arretiert. In der Tat stellt die α-CTD, neben der Erkennung von UP-Elementen, oft eine Interaktionsoberfläche mit Transkriptionsfaktoren dar. Neben den oben erwähnten Repressoren gibt es auch Aktivatoren, die über eine direkte Aktivator-α-CTD-Interaktion funktionieren, wie z. B. CcpA am *ackA*-Promotor oder SoxS während oxidativer Stressbedingungen (Turinsky *et al.*, 1998; Shah & Wolf, 2004; Kim *et al.*, 2005).

Am *gapB*-Promotor kann eine Interaktion mit der α-CTD aufgrund der Position der Operatoren und mit dem σ-Faktor aufgrund der Interaktionsexperimente ausgeschlossen werden, jedoch wäre eine Interaktion mit anderen Bereichen der RNAP möglich. Allerdings gibt es nur wenige Proteine, die mit der β- oder β'-Untereinheit interagieren. Ob CcpN tatsächlich mit anderen Teilen der RNAP wechselwirken kann, muss noch experimentell bestätigt oder widerlegt werden, aber die geringe Größe von CcpN, die wenig Platz für mehrere Interaktionsoberflächen bietet und die Tatsache, dass erhebliche Änderungen der DNA-Struktur durch die Bindung von CcpN auftreten, sprechen für die oben genannte Repression durch DNA-Strukturänderungen, wie sie z. B. für H-NS gezeigt werden konnte (Schneider *et al.*, 1999).

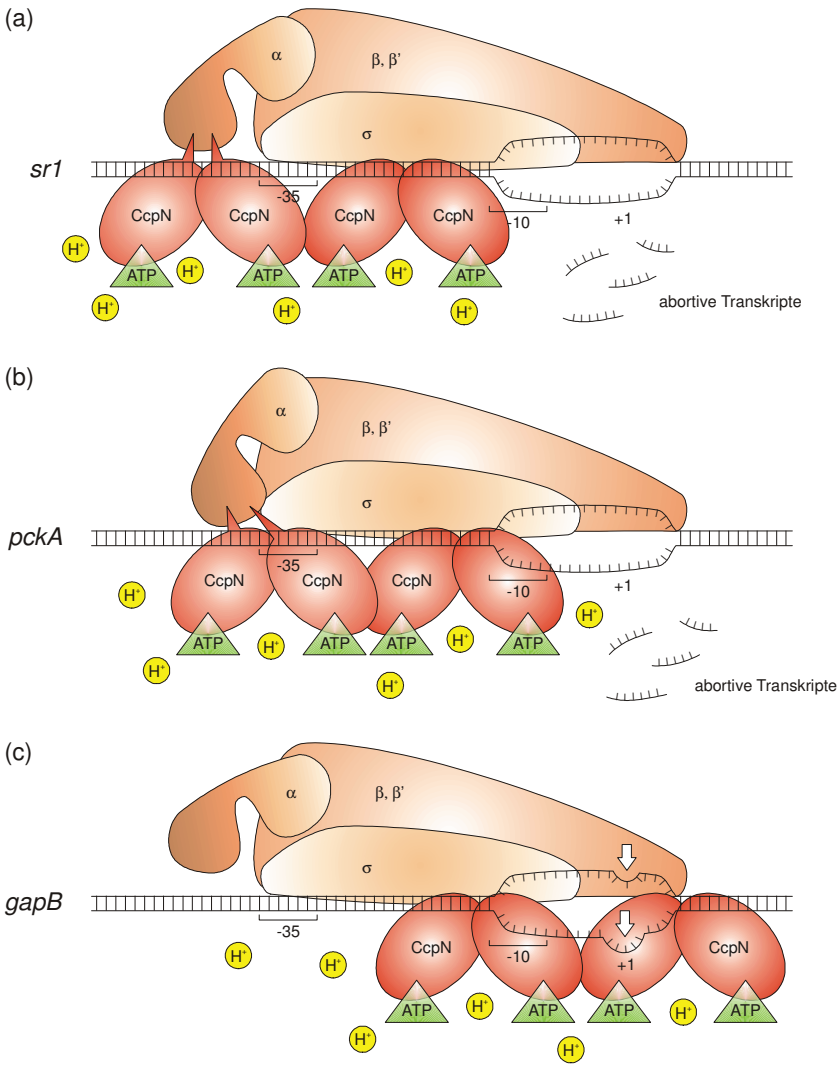

Abbildung 6:

Aktuelles Modell der CcpN-Aktivität unter glycolytischen Bedingungen

(a): Situation am *sr1*-Promotor. Sowohl CcpN als auch die RNAP sind an den Promotor gebunden. Durch die ablaufende Glycolyse ist der ATP-Spiegel der Zelle hoch, es ist nur wenig ADP vorhanden, sodass ATP an CcpN binden kann. Zusätzlich ist durch die Anreicherung von Acetat der intrazelluläre pH-Wert leicht gesenkt. CcpN kann mit der α-Untereinheit der RNAP interagieren, was noch die Synthese abortiver Transkripte erlaubt, aber nicht den Übergang in einen Elongationskomplex. Die von CcpN ausgehenden „Stacheln" symbolisieren die Interaktion mit der α-CTD.

(b): Situation am *pckA*-Promotor. Die Situation stellt sich hier, abgesehen von leicht veränderten Operator-Positionen, ähnlich der am *sr1*-Promotor dar.

(c): Situation am *gapB*-Promotor. Aufgrund der Position der Operatoren kann CcpN die α-Untereinheit nicht kontaktieren. Wahrscheinlich induziert CcpN hier Strukturänderungen in der DNA, die die Synthese abortiver Transkripte inhibieren. Weiße Pfeile zeigen Positionen, an denen DNA-Strukturänderungen festgestellt wurden, die mit der Transkription interferieren könnten. Als Alternative ist auch eine direkte Interaktion von CcpN und der β- oder β'-Untereinheit der RNAP denkbar.

Das Beispiel von CcpN zeigt eindrucksvoll, wie ein Repressor – bedingt durch variable Positionierung von Operatoren – auf verschiedene Mechanismen der Repression zurückgreifen kann. Variable Operator-Positionen, wie z. B. bei DeoR aus *E. coli*, sind nicht selten, allerdings gibt es wenige Belege dafür, dass diese auch mit verschiedenen Repressionsmechanismen einhergehen. Dies liegt allerdings vordergründig daran, dass für viele Repressoren der Repressionsmechanismus noch nicht aufgeklärt wurde. Ein gut dokumentiertes Beispiel hingegen ist der Transkriptionsfaktor CcpA aus *B. subtilis*, der in Abhängigkeit von der Operator-Position verschiedene Arten der Repression oder sogar der Aktivierung vermitteln kann (Kim *et al.*, 2005). Eine Positionierung des *cre*-Elements innerhalb des Promotors führt dabei, wie beim *amyE*-Gen, zu einer Behinderung der Bindung der RNAP (Nicholson *et al.*, 1987), *cre*-Elemente downstream des Promotors zu einer Blockierung der Elongation (Kim *et al.*, 2002) und eine Positionierung upstream des Promotors zu einer Aktivierung (Turinsky *et al.*, 1998).

Die Gesamtheit der vorliegenden Resultate gibt einen sehr genauen Einblick in die Regulation der Aktivität von CcpN und die von CcpN genutzten Repressionsmechanismen. Abbildung 6 zeigt eine Zusammenfassung der bisherigen Erkenntnisse unter Berücksichtigung des metabolischen Zustandes und der CcpN-Operatorpositionen an den drei untersuchten Promotoren.

7.4. Suche nach neuen CcpN-Zielgenen

7.4.1. Die intrazellulare Konzentration von CcpN und Suche nach neuen Zielgenen

Mittels Western-Blot wurde die Menge an CcpN-Molekülen in einer *B. subtilis*-Zelle auf ca. 4000 bestimmt. Diese recht hohe Konzentration erklärt die intensiven CcpN-DNA-Komplexe, die in EMSAs mit *B. subtilis*-Proteinrohextrakten detektiert werden konnten (Licht *et al.*, 2005). Die intrazelluläre Konzentration verschiedener Transkriptionsfaktoren reicht von 10-20 Molekülen pro Zelle beim LacI-Repressor (Lin & Riggs, 1975) bis hin zu 15000 Molekülen pro Zelle im Falle von CopR, einem Repressor, der die Kopiezahl des Streptokokken-Plasmids pIP501 reguliert (Steinmetzer *et al.*, 1998). CcpA oder CodY, zwei pleiotrope Regulatoren aus *B. subtilis*, kommen in Konzentrationen von ca. 3000 bzw. 2500 Molekülen pro Zelle vor (Miwa *et al.*, 1994; A. Sonenshein, persönliche Mitteilung) und weisen damit eine ähnlich hohe Konzentration wie CcpN auf. Interessanterweise scheint die Konzentration intrazellulär codierter Repressoren zumindest teilweise mit der Menge der von

ihnen regulierten Gene und Operons zu korrelieren: Während der LacI-Repressor nur ein Operon kontrolliert, regulieren CcpA und CodY jeweils ca. 100 Gene oder Operons (Sonenshein, 2005; Sonenshein, 2007). Diese Beobachtung war Ausgangspunkt, um neue Zielgene von CcpN zu suchen und zu identifizieren.

Dazu wurde im Genom von *B. subtilis* nach neuen CcpN-Operatoren gesucht und 22 mögliche Operatoren in Promotornähe entdeckt, die der Konsensussequenz entsprachen sowie 291 mögliche Operatoren, die eine Abweichung vom Konsensus aufwiesen. Von diesen Operatoren zeigten nur drei eine spezifische Bindung von CcpN: Diese lagen upstream der Gene *thyB*, *yhaM* und *gcaD*. Alle diese Operatoren banden CcpN teilweise deutlich schlechter als der starke *sr1*-Operator.

ThyB codiert eine Neben-Thymidylat-Synthase in *B. subtilis*, die für 5 % der Thymidylat-Synthese in diesem Organismus verantwortlich ist, die restlichen 95 % werden von ThyA synthetisiert (Neuhard *et al.*, 1978). *B. subtilis* stellt in dieser Hinsicht eine Besonderheit dar, da es der einzige Organismus ist, in dem zwei Thymidylat-Synthasen gefunden wurden. Die hier untersuchte ThyB ist ein Homologes der in anderen Bakterien vorkommenden Thymidylat-Synthase, während die aktivere ThyA der Thymidylat-Synthase aus Phagen ähnelt (Tam & Borriss, 1998). YhaM wurde als 5'-3'-Exoribonuclease identifiziert, die durch Magnesium- und Cobalt-Ionen aktiviert und durch Mangan-Ionen inhibiert wird (Oussenko *et al.*, 2002). Sie spielt vermutlich eine Rolle während der DNA-Replikation (Noirot-Gros *et al.*, 2002). GcaD codiert die N-Acetyl-Glucosamin-Pyrophosphorylase, ein Enzym, das an der Biosynthese von Peptidoglycan und Lipopolysacchariden und damit letztendlich am Zellwandaufbau beteiligt ist (Hove-Jensen, 1992). Da die Interaktion von CcpN mit den *yhaM*- und *gcaD*-Operatoren äußerst schwach war, wurde nur der *thyB*-Operator weiter untersucht.

7.4.2. CcpN-vermittelte Regulation von *thyB*

DNase I-Footprints zeigten, ähnlich wie am *pckA*-Promotor, nur eine zusammenhängende geschützte Region, die sowohl die –10- also auch die –35-Box umfasst. Interessanterweise war der Schutz vor DNase I-Abbau über den gesamten Footprint nicht konstant wie am *sr1*-, *gapB*- und *pckA*-Promotor, sondern innerhalb der Konsensus-Sequenz deutlich intensiver. Auch Interferenz-Footprints zeigten eine neuartige Kontaktverteilung am *thyB*-Promotor mit den stärksten Kontakten innerhalb des Konsenus-Operators und schwächeren Kontakten in zwei zusätzlichen Operatoren, während an allen anderen CcpN-regulierten Promotoren nur

eine starke und eine schwache Bindungsstelle vorhanden ist. Zum am weitesten upstream gelegenen Operator am *thyB*-Promotor werden fast ausschließlich Kontakte zu Adenin-Resten ausgebildet. Da diese Interferenz-Footprinting-Methode Kontakte zu Adeninen in der kleinen Furche detektiert, kann man daraus schlussfolgern, dass CcpN hier einen DNA-Kontakt über die kleine Furche herstellt. Kontakte in der kleinen Furche finden oft in Form von indirektem Auslesen, d. h. Erkennung von DNA-Strukturen anstatt Sequenzen, statt, wie z. B. bei CRP oder HU in *E. coli* (Swinger & Rice, 2007; Lindemose *et al.*, 2008), oder dienen oft nur der Stabilisierung von Protein-DNA-Interaktionen, da die kleine Furche nur einen geringen Informationsgehalt aufweist.

LacZ-Transkriptionsfusionen und *in vitro*-Transkriptions-Versuche zeigten, dass CcpN am *thyB*-Promotor als schwacher Aktivator wirken kann. Die nur geringe Aktivierung an diesem Promotor – verglichen mit der starken CcpN-vermittelten Repression an anderen Promotoren – kann durch die verhältnismäßig schwache Bindung von CcpN an den Operator des *thyB*-Promotors erklärt werden.

Da Glycolyse in der Regel mit exzellenten Wachstumsbedingungen und einer erhöhten Wachstumsrate einhergeht, erscheint eine Erhöhung der Thymidylat-Synthase-Aktivität physiologisch sinnvoll. Allerdings trägt ThyB nur 5 % zur gesamten Thymidylat-Synthese bei, was eine Erhöhung um 50 % als verschwindend gering erscheinen lässt. Es ist jedoch durchaus denkbar, dass ThyB die ursprüngliche Thymidylat-Synthase der Vorfahren von *B. subtilis* war, deren Aktivität im Laufe der Evolution verloren ging, während die Regulation zumindest teilweise intakt blieb. Um diese Hypothese zu untersuchen, wurde ein Alignment der Promotor-Regionen der *thyB*-Gene verschiedener Firmicuten, die CcpN-Homologe codieren, durchgeführt. In zahlreichen der untersuchten Organismen wurden Sequenzen, die einer CcpN-Konsensus-Sequenz ähnelten, upstream des Start-Codons des jeweiligen *thyB*-Gens identifiziert, in den Spezies *B. amyloliquefaciens*, *B. anthracis*, *B. cereus*, *S. aureus* und *F. nucleatum* sogar in nahezu gleichem Abstand wie in *B. subtilis*. In den CcpN-Operator-Homologen anderer Organismen wurden mehr Mismatches gefunden, jedoch ist nicht auszuschließen, dass in diesen Organismen andere Sequenzanforderungen an CcpN-Operatoren bestehen. Da in allen diesen Organismen ThyB die einzige Thymidylat-Synthase ist, ist durchaus denkbar, dass das *thyB*-Gen hier von CcpN reguliert wird und die CcpN-vermittelte Regulation tatsächliche physiologische Relevanz besitzt.

8. Zusammenfassung/Summary

8.1. Zusammenfassung

Im Rahmen dieser Arbeit wurde der Transkriptionsfaktor CcpN aus dem Gram-positiven Bakterium *Bacillus subtilis*, der die Gene *sr1*, *pckA* und *gapB* reguliert, eingehend charakterisiert.

Dazu wurden zunächst mittels Interferenz-Footprinting am *sr1*-, *pckA*- und *gapB*-Promotor die Basen der Operatoren bestimmt, die für eine Kontaktierung durch CcpN nötig sind. Dabei wurde zum einen festgestellt, dass besonders intensive Kontakte zu dem Bereich ausgebildet wurden, der der vorher bestimmten Konsensus-Sequenz für CcpN entspricht, zum anderen, dass an den untersuchten Promotoren jeweils zwei unterschiedlich stark kontaktierte Operatoren existieren. Es wurde gezeigt, dass sich die unterschiedliche Kontaktstärke der Operatoren auch in einer unterschiedlich starken Bindung der Einzeloperatoren in EMSAs niederschlägt, wenn nur einer der Operatoren auf einem DNA-Fragment vorhanden war. Waren beide Operatoren vorhanden, was der *in vivo*-Situation entspricht, wurden diese gleich stark und außerdem intensiver als die Einzeloperatoren gebunden. Anhand der Energiebilanz der CcpN-Operator-Interaktionen und der Veränderung der Bindungskurve beim Vergleich von zwei isolierten Operatoren mit zwei Operatoren auf einem DNA-Fragment wurde berechnet, dass die Bindung von CcpN an seine Operatoren kooperativ geschieht. Des Weiteren wurden energetische Betrachtungen der CcpN-DNA-Interaktion bei verschiedenen von *B. subtilis* tolerierten Temperaturen durchgeführt und dabei festgestellt, dass die CcpN-DNA-Interaktion primär enthalpisch getrieben ist.

Da CcpN in konstanter Menge in der Zelle vorhanden und seine Expression nicht reguliert ist, wurde nach intrazellulären Effektoren für CcpN gesucht. Es konnte gezeigt werden, dass CcpN auch ohne Effektoren die drei untersuchten Promotoren spezifisch, jedoch unreguliert, reprimieren kann, was eine Erklärung für die starke Derepression der CcpN-regulierten Gene in einem *ccpN*-Knockout-Stamm liefert. Weiterhin konnten mit Hilfe von *in vitro*-Transkriptions-Versuchen ATP und niedriger pH-Wert als positive Effektoren von CcpN identifiziert werden. Diese Ergebnisse fügen sich gut in die beobachtete Aktivität von CcpN *in vivo* ein, das nur unter glycolytischen Bedingungen als Repressor wirkt, welche in der Regel mit hohem ATP-Spiegel und leicht gesenktem pH-Wert aufgrund der Acetat-

Produktion einhergehen. Für ADP hingegen wurde gezeigt, dass es dem aktivierenden Effekt von ATP entgegenwirken kann. Beide Befunde wurden durch CD-Messungen unterstützt, die eine deutliche Strukturänderung von CcpN bei ATP-Bindung im Sauren, jedoch nicht bei neutralem pH-Wert zeigten und zudem nur eine schwache Strukturänderung bei ADP-Bindung sowohl im Sauren als auch im Neutralen. Daraus wurde geschlussfolgert, dass die Repressionsaktivität von CcpN durch ATP-induzierte Strukturänderungen erhöht und durch die Bindung von ADP wieder gesenkt werden kann.

Da in der CcpN-Sequenz zwei CBS-Domänen existieren, für die in der Vergangenheit die Bindung von Adenosin-Resten gezeigt werden konnte, wurde eine CcpN-Mutante untersucht, bei der eine konservierte Aminosäure innerhalb einer CBS-Domäne durch ein Alanin ausgetauscht wurde. Diese Mutante, die dieselbe Struktur wie der Wildtyp besaß und auch noch in der Lage war, DNA zu binden, reagierte in der *in vitro*-Transkription nicht mehr auf ATP, wodurch gezeigt wurde, dass die CBS-Domänen auch bei CcpN für die ATP-Bindung verantwortlich sind.

Letztendlich wurde noch mit Hilfe von EMSAs demonstriert, dass ATP und niedriger pH-Wert die Affinität von CcpN zu seinen Operatoren nicht ändern können.

Da bisher nichts über den Repressionsmechanismus von CcpN bekannt war, sollte dieser an den drei vorher untersuchten Promotoren aufgeklärt werden. Mittels EMSAs konnte gezeigt werden, dass CcpN und die RNA-Polymerase gleichzeitig an die Promotorregionen binden können und dass die Bindungsintensität unter Repressionsbedingungen nicht abnimmt. Allerdings konnte eine Konkurrenz in der Bindung von CcpN und RNAP festgestellt werden, welche die relativ starke konstitutive Repression CcpN-regulierter Gene *in vivo* erklärt. „Open complex formation“-Assays zeigten, dass an allen drei untersuchten Promotoren auch unter Repressionsbedingungen ein offener Komplex ausgebildet werden konnte und CcpN somit diesen Schritt der Transkriptionsinitiation nicht reguliert. Am *gapB*-Promotor konnten unter Repressionsbedingungen keine abortiven Transkripte mehr detektiert werden, was auf eine Inhibition der Bildung des Transkriptionsinitiationskomplexes hinweist. Am *sr1*- und *pckA*-Promotor hingegen waren abortive Transkripte noch nachweisbar, allerdings wurde an diesen beiden Promotoren der Übergang in den Elongationskomplex inhibiert.

Interaktionsstudien mittels Far-Western-Blot und Co-Elutions-Versuchen zeigten, dass CcpN in der Lage ist, mit der α-Untereinheit der RNAP, nicht jedoch mit dem σ-Faktor, zu interagieren. Dies legte nahe, dass die Inhibition des Übergangs in den Elongationskomplex am *sr1*- und *pckA*-Promotor durch Arretierung der RNAP am Promotor, vermittelt durch eine

Interaktion zwischen CcpN und der α-Untereinheit, erfolgt. Am *gapB*-Promotor besteht diese Möglichkeit aufgrund der Position der Operatoren nicht, doch wurde an diesem Promotor in vorangegangenen Arbeiten eine starke Änderung der DNA-Struktur bei der Bindung von CcpN gezeigt, durch die die Transkriptionsinitiation blockiert werden könnte.

Bereits eine erste Charakterisierung von CcpN deutete an, dass die intrazelluläre Konzentration relativ hoch sein muss. Dies wurde in dieser Arbeit mittels Western-Blot verifiziert und die Menge an CcpN in einer *B.-subtilis*-Zelle auf ca. 4000 Moleküle bestimmt. Da eine gewisse Korrelation zwischen der Menge eines Transkriptionsfaktors und der Zahl der regulierten Gene besteht, wurde im Genom von *B. subtilis* nach weiteren Zielgenen für CcpN gesucht. Die Suche nach CcpN-Operatoren ergab zahlreiche Treffer, von denen allerdings nur diejenigen im Promotorbereich der Gene *thyB*, *yhaM* und *gcaD* eine Bindung von CcpN *in vitro* zeigten. Die Interaktion mit dem Operator des *thyB*-Promotors wurde als einzige näher untersucht, da die beiden anderen Operatoren nur eine schwache Interaktion mit CcpN zeigten. Dabei wurde ein bisher nicht beobachtetes Bindungsmuster festgestellt, bei dem ein starker Operator und zwei schwache ohne Spacer direkt aneinandergrenzen.

Mit Hilfe von *lacZ*-Transkriptionsfusionen und *in-vitro*-Transkriptionen konnte gezeigt werden, dass CcpN am *thyB*-Promotor unter glycolytischen Bedingungen einen schwachen aktivierenden Effekt ausübt. Diese Aktivierung scheint physiologisch wenig bedeutsam, da das *thyB*-Gen in *B. subtilis* kaum genutzt wird, weil seine Aktivität fast vollständig von *thyA* übernommen wurde. Im Gegensatz dazu besitzen andere Bakterien nur ein *thyB*-Gen-Homologes. Interessanterweise wurden in den *thyB*-upstream-Regionen verschiedener CcpN-codierender Spezies durch Alignments CcpN-Operator-Sequenzen entdeckt, die darauf hindeuten, dass in diesen Spezies ebenfalls eine Regulation von *thyB* durch CcpN stattfinden könnte.

Insgesamt konnten mit den in dieser Arbeit vorgestellten Ergebnissen die Erkenntnisse über den Transkriptionsfaktor CcpN aus *B. subtilis* stark erweitert werden. Zusammen mit früheren Arbeiten wird CcpN dadurch zu einem der bestcharakterisierten Transkriptionsfaktoren in *B. subtilis* und zu einem der wenigen, für die der Repressionsmechanismus aufgeklärt wurde.

8.2. Summary

The scope of this work was the detailed characterisation of the transcription factor CcpN of the Gram-positive bacterium *B. subtilis* which regulates the genes *sr1*, *pckA* and *gapB*.

To this end, bases within the operators that are necessary for forming contacts with CcpN were determined at the *sr1*, *pckA* and *gapB* promoters by interference footprinting. These experiments showed that intensive contacts were made within a sequence corresponding to the previously determined consensus sequence and that each promoter consists of two operator segments with different contact strength. EMSAs demonstrated that these differences in contact strength also resulted in a varying binding strength of the single operators when present on a separate DNA fragments. However, if both operators were located on one DNA fragment, both were bound with equal strength and in addition more intensively than the single operator sites. Regarding the change in shape and slope of the binding curves when comparing single operators with operator pairs and the energy gain resulting from CcpN-operator interaction it was concluded that CcpN binds its operator sites cooperatively. Furthermore, energetic calculations of CcpN-DNA interaction at different temperatures revealed that the binding process is driven by a strong enthalpy rather than strong entropy, ensuring a stable interaction of CcpN with its operators over a large temperature scale.

Since CcpN is present in constant concentrations within the cell and the expression of its gene was found to be not regulated, a search for intracellular effectors of CcpN was performed. *In vitro* transcription reactions showed that purified CcpN without an effector is able to specifically repress transcription at the three investigated promoters and provides an explanation for the strong derepression observed in a *ccpN* knockout strain. ATP and low pH were identified as the intracellular activators of CcpN activity, fitting quite well into CcpN's scheme of action: During glycolysis, when CcpN is active, ATP levels in the cell are high and the cytosol becomes slightly acidic due to acetate production. On the contrary, ADP has been shown to counteract the activating effect of ATP at equimolar concentrations. Both results have been substantiated by CD spectroscopy, which showed extensive structural rearrangements of CcpN upon ATP binding at low pH, but not at neutral pH, while ADP binding did only result in weak structural alterations. It was thus concluded that the repression activity of CcpN is stimulated by structural alterations induced by ATP binding at low pH and repressed by ADP binding.

Two CBS domains, which are able to bind adenosine residues, have been found within the CcpN sequence. To elucidate the role of these domains, a CcpN mutant with an amino acid exchange in a conserved residue in one domain was investigated. This mutant, although still being able to bind to DNA and showing the same structure as the wild type, did no longer respond to ATP, indicating that the CBS domains are indeed responsible for ATP binding.

Eventually, EMSAs performed under repressing and non-repressing conditions showed that the positive effectors of CcpN did not alter the affinity to its operators.

Since nothing was known about the repression mechanism of CcpN, efforts have been made to elucidate this mechanism at the three investigated promoters. EMSAs have demonstrated that CcpN and RNAP are able to bind together to the promoter under repressive and non-repressive conditions. However, competition for promoter binding between these two proteins has been observed which explains the strong derepression in a *ccpN* knockout strain. Using open complex formation assays it has been demonstrated that open complexes can still be formed under repressive conditions at all three promoters, showing that CcpN does not prevent melting of the DNA. At the *gapB* promoter, no abortive transcripts were detectable under repressive conditions indicating that CcpN represses transcription initiation at this promoter. The *sr1* and *pckA* promoters still showed abortive transcript synthesis under repressive conditions, but the transition to the elongation complex was inhibited.

Far western blot and co-elution interaction studies showed that CcpN is able to specifically interact with the α-subunit of RNAP, but not with the σ-factor. This suggests that the repression at the *sr1* and *pckA* promoters occurs by interaction between CcpN and the α-subunit, which in turn stalls the RNAP at the promoter. This mechanism can, however, be excluded for the *gapB* promoter, since the operator location does not allow CcpN to be positioned in a way to properly contact the α-subunit. Here, an alteration in DNA structure upon CcpN binding has been detected in preceding investigations, which is potentially responsible for the prevention of transcription initiation.

Early characterisations of CcpN already suggested a rather high intracellular concentration. This observation was substantiated using western blots, and the amount of CcpN was determined to be 4000 molecules per cell. Since there is a certain correlation between repressor concentration and the number of regulated genes, a search for new CcpN target genes was performed in the genome of *B. subtilis*. The search resulted in numerous potential operators, but only those located in the promoter region of the *thyB*, *yhaM* and *gcaD* genes

showed CcpN binding *in vivo*, with *thyB* being the only one strong enough to be investigated further. Interestingly, the *thyB* CcpN operator showed a binding pattern not observed before, where one strong and two weak operators are conjoined without a spacer region.

Using *lacZ* transcriptional fusions and *in vitro* transcription, a slight activation of the *thyB* promoter by CcpN under glycolytic conditions has been shown. This regulation seems to be of little physiological relevance in *B. subtilis*, since the *thyB* gene is hardly active, and thymidylate synthase activity is mainly carried out by ThyA. However, related species containing only a *thyB* and no *thyA* gene have CcpN operator sequences upstream of their respective *thyB* genes and it is feasible that CcpN plays an important regulatory role in *thyB* expression in these bacteria.

The results of the presented study greatly increase our understanding of the transcription factor CcpN from *B. subtilis*. This makes – together with preceding works – CcpN to one of the best characterised transcription factors in *B. subtilis* including one of the few for which a repression mechanism has been determined.

9. Literaturverzeichnis

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Selbstständigkeitserklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Jena, 14.08.2009

Erklärung zur Bewerbung

Ich erkläre, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad Dr. rer. nat. beworben habe und dass ich weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des o. g. akademischen Grades an einer anderen Hochschule beantragt habe.

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Publikationen und Tagungsbeiträge

Publikationen:

1. Licht, A., Preis, S. & Brantl, S. (2005) Implication of CcpN in the regulation of a novel untranslated RNA (SR1) in *Bacillus subtilis*. *Mol. Microbiol.* **58**: 189-206.
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Tagungsbeiträge:

1. Licht, A. & Brantl, S. (2006) High resolution contact probing between *Bacillus subtilis* transcriptional repressor CcpN and its operators: A comparison at the molecular level. Poster bei der „Jahrestagung der Vereinigung für allgemeine und angewandte Mikrobiologie“, Jena, Deutschland.
2. Licht, A. & Brantl, S. (2006) Identification and characterisaton of CcpN, a novel sugar dependent transcriptional repressor from *Bacillus subtilis*. Vortrag beim „26th Symposium on Mechanisms of Gene Regulation“, Königswinter, Deutschland.
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Thesen zur Dissertation

1. Im Rahmen dieser Arbeit wurde der Transkriptionsfaktor CcpN aus dem Bakterium *Bacillus subtilis* eingehend charakterisiert.
2. CcpN besetzt an den Promotoren der Gene *sr1*, *pckA* und *gapB* jeweils zwei Operatoren, die zwei (am *pckA*-Promotor) oder drei (am *sr1*- und *gapB*-Promotor) Helixwindungen auseinander liegen.
3. Einer der Operatoren wird dabei jeweils stärker kontaktiert und – wenn isoliert vorhanden – auch stärker gebunden als der andere, wobei die Sequenz dieses Operators in allen Fällen deutlich näher an der Konsensussequenz als die des anderen Operators ist.
4. Sind beide Operatoren auf einem DNA-Fragment vorhanden, werden sie von CcpN gleich stark gebunden, die dabei im Vergleich zur Bindung der Einzeloperatoren freiwerdende Energie und die Änderung der Bindungskurven zeigen, dass CcpN die DNA kooperativ bindet.
5. Die Bestimmung der freien Bindungsenergie bei unterschiedlichen Temperaturen zeigt, dass die CcpN-DNA-Interaktion enthalpisch getrieben wird.
6. Da die Expression von CcpN nicht reguliert wird, muss es einen intrazellulären Regulator geben, der die Aktivität in Abhängigkeit von der Stoffwechselsituation modifiziert.
7. CcpN kann in hoher Konzentration auch ohne Regulator die Expression am *sr1*-, *pckA*- und *gapB*-Promotor spezifisch reprimieren.
8. ATP und niedriger pH-Wert sind die intrazellulären Effektoren von CcpN und erhöhen dessen Repressionsaktivität erheblich, ADP kann dem Effekt von ATP entgegenwirken und senkt die Repressionsaktivität von CcpN.
9. Bei der Bindung von ATP im Sauren ändert sich die Struktur von CcpN erheblich, jedoch nicht bei der Bindung im Neutralen oder bei der Bindung von ADP.
10. CcpN mit einer Mutation in einer CBS-Domäne weist dieselbe Struktur auf wie der Wildtyp und ist noch in der Lage, DNA zu binden, reagiert allerdings nicht mehr auf ATP. Dies zeigt, dass die CBS-Domäne für die ATP-Bindung zuständig ist.
11. Die Anwesenheit von ATP bei saurem pH-Wert ändert die Affinität von CcpN zur DNA nicht.
12. CcpN kann auch unter reprimierenden Bedingungen gemeinsam mit der RNA-Polymerase an den *sr1*-, *pckA*- und *gapB*-Promotor binden, konkurriert aber sowohl

unter reprimierenden als auch unter nicht-reprimierenden Bedingungen in gleicher Weise mit der RNAP um die Bindungsstellen des Promotors, was die starke Derepression in einem *ccpN*-Knockout-Stamm erklärt.

13. CcpN ist nicht in der Lage, die Bildung von offenen Komplexen an den drei untersuchten Promotoren zu verhindern.
14. Am *gapB*-Promotor werden unter reprimierenden Bedingungen keine abortiven Transkripte mehr gebildet, da CcpN hier die Transkriptionsinitiation blockiert.
15. Am *sr1*- und *pckA*-Promotor wird auch unter reprimierenden Bedingungen die Transkription initiiert, allerdings verhindert CcpN den Übergang in einen Elongationskomplex.
16. CcpN ist in der Lage, spezifisch mit der α-Untereinheit der RNA-Polymerase zu interagieren und nutzt diese Interaktion wahrscheinlich, um die RNAP am *sr1*- und *pckA*-Promotor zu arretieren.
17. CcpN kann am *gapB*-Promotor aufgrund der Lage der Operatoren nicht mit der α-Untereinheit interagieren und blockiert die Transkriptionsinitiation hier vermutlich durch Änderungen in der DNA-Struktur.
18. CcpN ist in einer Kopiezahl von 4000 Molekülen pro Zelle vorhanden.
19. Im Genom von *B. subtilis* existieren ca. 300 potentielle CcpN-Bindungsstellen upstream von Genen, von denen neben den drei bisher bekannten die in der Promotorregion der Gene *thyB*, *yhaM* und *gcaD* Bindungsaktivität zeigen.
20. Der *thyB*-Promotor zeigt eine neuartige Operatorverteilung, bei der eine Hauptoperator und zwei Hilfsoperatoren ohne Spacer aneinandergrenzen.
21. CcpN kann als schwacher Aktivator am *thyB*-Promotor wirken.
22. In anderen Spezies, die CcpN codieren, finden sich CcpN-Operatoren upstream des entsprechenden *thyB*-Gens, was auf eine Regulation durch CcpN auch in diesen Bakterien hindeutet.